STUDIES ON THE IN VIVO SECRETION AND METABOLISM OF THE STEROID HORMONES OF THE ADRENAL CORTEX

by

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List of publications.

1. Holzbauer, M. and Vogt, M. 1961. Corticosteroids in plasma and cells of adrenal venous blood. J. Physiol. 157, 137 - 156.

2. Holzbauer, M. and Vogt, M. 1966. Investigations into the causes of the rise in aldosterone secretion during haemorrhage. Parts I and II. Phil. Trans. R. Soc. London, 250, 243 - 310.

3. Holzbauer, M. 1964. The part played by ACTH in determining the rate of aldosterone secretion during operative stress. J. Physiol. 172, 138 - 149.

4. Holzbauer, M. and Vogt, M. 1964. Observations on slow rhythmic blood pressure waves (Mayer waves) in the dog. J. Physiol. 172, 5 - 7P.

5. Heap, R. B. and Holzbauer, M. 1965. Gas chromatography of androgens, progesterone and progesterone derivatives in adrenal venous blood of pigs and dogs. J. Physiol. 183, 11P.

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8. Holzbauer, M. and Newport, H. M. 1968. Secretion of 3β-hydroxypregn-5-en-20-one (pregnenolone) by the adrenal gland. Nature, 217, 967 - 968.

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16. Rembeisa, R., Holzbauer, M., Young, P. C. M., Birmingham, M. K. and Saffran, M. 1967. Metabolism of 17α-methylandrostenediol and 17β-methyltestosterone by the rat adrenal gland in vitro. Endocrinology, 81, 1278 - 1284.

Summary

This thesis consists of publications describing experiments in which the secretion of steroid hormones by the adrenal cortex was studied. The venous effluent from the adrenal glands of several mammalian species was collected under anaesthesia. Chemical methods were developed which allow the simultaneous qualitative and quantitative analysis of most steroid hormones synthesized by the adrenal cortex. These methods were applied to blood and tissue extracts. Because of the observation (paper 1) that a large proportion of the steroids in the blood is loosely associated with blood cells, whole blood samples were extracted instead of plasma alone. The experiments have given information on the type of steroids secreted by the adrenal cortex and on factors which influence the rate at which they are secreted.

One group of papers is concerned with the control of aldosterone secretion in the dog (papers 2, 3 and 4). A detailed study of the factors which cause a rise in aldosterone secretion following acute haemorrhage (paper 2) led to the conclusion, that acute blood loss stimulates the release of hormones from the pituitary gland and the kidney. These hormones in turn cause increased aldosterone secretion. In this respect the two organs can replace each other. In dogs which prior to the experiment had been maintained for long periods of time on either very low or very high dietary sodium intake it was usually not possible to observe the aldosterone rise after blood loss.

The aldosterone stimulating substance released from the pituitary gland is in all probability ACTH. This is strongly supported by a quantitative study in which aldosterone secretion was

measured in the same dog before and after hypophysectomy and during subsequent infusions of ACTH (paper 3). The increase in aldosterone secretion was found to depend on the dose of ACTH, provided aldosterone secretion was not maximally stimulated by factors not of pituitary origin. In the same experiments information was obtained on the rate at which ACTH was secreted during anaesthesia and operative stress, by comparing glucocorticoid secretion rates before hypophysectomy with those after hypophysectomy, when ACTH was infused at different rates.

The condition of the circulation before and after haemorrhage was found to be important for the ability of a dog to respond to blood loss with a rise in aldosterone secretion. The occurrence of a certain type of blood pressure waves (Mayer waves) is indicative of circulatory impairment and a study was made of the nature of such waves (paper 4).

A second group of papers (5 - 13) is concerned with a class of corticosteroids which has so far not been studied in a systematic and quantitative manner, mainly because they are only secreted in small quantities and methods for their estimation had not been available. The papers describe the development and adaptation of paper and gas-chromatographic techniques for the purpose of a qualitative and quantitative study of these steroids.

Pregnenolone, progesterone, 17αOH-progesterone, 11βOH-progesterone and the three so called adrenal androgens androstenedione, adrenosterone and 11βOH-androstenedione were consistently found to be present in the extracts of adrenal venous blood of dogs and young pigs in concentrations similar to or higher than those of aldosterone. Hypophysectomy caused a fall in the secretion of these steroids, similar to that of the glucocorticoids (papers 6, 8 and 9) but their secretion did not cease completely, indicating that these steroids are not only secreted by an overactive gland under conditions of

stress but also under resting conditions. In addition to the above steroids, 16αOH-progesterone was found to be secreted by the adrenal gland of the young pig(7). Certain experimental conditions modified the secretion of pregnenolone and 11βOH-androstenedione in a different way from that of other steroids (paper 10). Studies of this type may eventually help to explain certain clinical signs of adrenal deficiency and overactivity which cannot fully be attributed to the lack or excess of glucocorticoids or aldosterone.

The quantities of progesterone secreted by the adrenal gland of the pig (papers 5, 6 and 10) and the rat (paper 11) can be of the same order as those secreted by the ovaries of the same species (paper 12) under similar experimental conditions.

A comparison between the rates at which steroids are secreted with the concentrations in which they are present in the adrenal tissue of one and the same animal provides some information on the rates of steroid synthesis in vivo (papers 9 and 10). The quantity of a given steroid present in the adrenal of a pig or dog was found to correspond to the amount secreted within 0.5 to five minutes. Pregnenolone and progesterone were exceptions to this rule. Assuming that these two steroids are the most important precursors of all the steroids secreted by the adrenal, it can be calculated, that the amounts at which they are present in the adrenal will be utilized within 1 - 5 minutes. Any stimulus leading to an increased secretion of adrenal steroids must therefore effect an increase in the rate at which pregnenolone is formed. In accordance with this, it was found that in the rat (paper 13) stress does not only cause a rise in the adrenal concentration of corticosterone but also of pregnenolone and progesterone.

The papers 10, 14 and 15 contain information on the possible use of drugs to prevent the release of ACTH caused by anaesthesia and the operative procedures required for adrenal vein cannulation. Chlorpromazine and morphine were not able to overcome this severe stress in the rat (papers 14 and 15). In dogs anaesthetised with sodium pentobarbitone in which the left adrenal vein had been cannulated, α-ethyltryptamine had an effect on steroid secretion similar to that of hypophysectomy. However it lacked this effect in dogs anaesthetised with chloralose and in dogs which had been eviscerated(paper 10).

The last paper (16) deals with the mechanism by which 17α-methylandrostenediol inhibits corticosterone production by the rat adrenal.

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CORTICOSTEROIDS IN PLASMA AND CELLS OF ADRENAL VENOUS BLOOD

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Investigations by several authors appeared to show that no appreciable amounts of corticosteroids are present in the cell fraction of adrenal venous blood (Bibile, 1953) or in peripheral human blood (Eik-Nes, Nelson & Samuels, 1953; Migeon, Sandberg, Decker, Smith, Paul & Samuels, 1956; Bush, 1957). This view has been generally accepted and as a result most of the estimates of the secretory capacity of the adrenal cortex have been carried out by studying the steroid content of the plasma alone. There are, however, hints in the literature that corticosteroids might become associated with the blood cells (Peterson, Wyngaarden, Guerra, Brodie & Bunim, 1955; Bush, 1957).

Studies on the secretion rate of aldosterone are made difficult by the very low concentrations of this steroid in adrenal venous plasma. It seemed, therefore, desirable to examine the possibility that some aldosterone was associated with the cells. A sample of adrenal venous blood was divided into two equal portions. One of these was centrifuged and the plasma and cells were extracted separately. The other portion was extracted as whole blood. The experiment showed that some aldosterone was, in fact, extracted from the cells, so that by using plasma alone a valuable source of aldosterone was wasted. It also showed that the estimate of aldosterone in the whole-blood sample was approximately three times as large as the sum of the estimates of aldosterone in the separated plasma and cell fractions of the same blood. This suggested that the method of extraction used broke down when applied to the closely packed cell fraction.

These observations have been followed up and the experiments have been extended to include estimations of corticosterone and cortisol.

METHODS

Operative procedures

Mongrel dogs were anaesthetized with ether followed by chloralose (70 mg/kg I.V.). The left adrenolumbar vein was exposed by an abdominal mid-line incision and cannulated

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lateral to the adrenal gland, after tributaries other than those coming from the adrenal gland had been tied. Heparin (500 u./kg) was injected intravenously. The adrenal vein was ligated at its point of entry into the vena cava. Adrenal venous blood was collected over periods of 20–150 min in polythene centrifuge tubes or glass cylinders immersed in ice-water.

When homologous blood or dextran was infused in the course of the experiment the dog received a preliminary intravenous injection of mepyramine maleate B.P. 5 mg/kg. This was done in order to try to prevent sudden falls in blood pressure and loss of plasma or dextran into the tissues. Bliss, Johns & Burgen (1959) and Remington & Baker (1959) have reported that dogs are often intolerant of homologous blood; oedema, loss of plasma from the circulation and prolongation of bleeding and clotting times may result from such infusions. Some of these effects are antagonized by mepyramine, but the improvement achieved is very variable.

Rat blood was collected under pentobarbitone anaesthesia (1 % solution, 45 mg/kg, I.P.), guinea-pig blood under sodium barbitone (15% solution, 600 mg/kg, I.P.), rabbit blood under urethane (25 % solution in 0·9 % NaCl, 7 ml./kg, I.V.).

Rat and guinea-pig adrenal venous blood was collected from the left renal vein after tying off the renal pedicle and all veins not coming from the adrenal. The guinea-pig was previously eviscerated. The rabbit was eviscerated and blood was collected from the inferior vena cava caudal to the entry of the right and left renal veins. The renal veins and arteries and the abdominal aorta were tied and the vena cava was occluded at the level of the diaphragm (Vogt, 1955).

Chemical procedures

Solvent purification. Ethyl acetate was shaken once with a 1 % solution of sodium carbonate and twice with water, dried over $CaCl_2$ and distilled twice. Methanol was distilled once from solid NaOH and redistilled. Ether was redistilled immediately before use.

Extraction. One volume of blood or plasma was diluted with one volume of water and extracted three times by shaking vigorously with two volumes of a mixture of ethyl acetate-ether 2:1 (v/v). Blood cells were first extracted in the same way. When this proved unsatisfactory they were diluted with three volumes of water instead of one. The amount of ethyl acetate-ether mixture used for each extraction was then four times the volume of the packed cells.

Purification of extracts. The purification procedure was similar to that described by Bush (1952) and by Holzbauer (1957). The combined extracts were washed consecutively with 1/8 of their calculated volume of a 0·2 N solution of Na_2CO_3, water and acidified water (10 drops of glacial acetic acid/100 ml.). The combined washings were twice re-extracted with 1/4 of their volume of the ethyl acetate-ether mixture. The washed extracts were dried with Na_2SO_4, decanted off and evaporated to dryness under reduced pressure at 50° C. For this purpose the flask was clamped into a device which was kept in slow rotating motion by a motor. The ethyl acetate-ether used to re-extract the washing solutions was poured over the Na_2SO_4 deposit, decanted and added to the main extract for evaporation. The dry residue of the combined extracts was then freed from fats by distribution between petroleum ether (b.p. 40–60° C) and ethanol. The volume of petroleum ether was 60 ml.; it was extracted once with 6 ml. and three times with 3 ml. of 80% ethanol. The ethanol was evaporated *in vacuo* in two portions from a 150 ml. round-bottom flask, with fresh petroleum ether added to reduce the often very troublesome frothing. The phospholipids were precipitated from acetone with $MgCl_2$, precisely as described before (Holzbauer, 1957).

Chromatography

All chromatograms were run at 27° C on Whatman No. 2 paper. The steroid spots were located on the developed papers by scanning in front of an U.V. lamp emitting at 240 mμ. The papers carried markers of pure steroids on each side.

Aldosterone, first chromatogram. After evaporation of the acetone the extract was dissolved

in 2 ml. ethanol and 1/20 of this solution removed for the estimation of cortisol and corticosterone. The remainder was evaporated to dryness, dissolved in 0·4 ml. dichloromethane and applied along a 5 cm horizontal line to a paper sheet, 22·5 cm wide. Two washings of 0·2 ml. CH_2Cl_2 were applied to the same region. A descending chromatogram was then run in the E_2B system of Eberlein & Bongiovanni (1955) which consists of petroleum ether (b.p. 80–100° C): tertiary butanol: water, 417:208:375 (v/v). In this system corticosterone has the highest R_F value, followed by cortisone and cortisol. The R_F value of aldosterone is somewhat smaller still than that of cortisol. Adrenal vein blood, however, contains so much cortisol that it invades the aldosterone region. (If 400 μg cortisol and 10 μg aldosterone were applied in this way, making sure that the area in which the two steroids were applied did not exceed 1 cm in the direction of the flow of the solvents, the small aldosterone spot could be clearly distinguished from, though it was touching, the large spot of cortisol.) For elution, rectangles of about 9×12 cm containing the aldosterone, contaminated by cortisol, were cut from the paper.

Aldosterone, acetylation. In order to separate aldosterone from cortisol the eluate was acetylated. The dried residue of the eluate was dissolved in 0·04 ml. of pyridine (purified by refluxing over BaO and distilling twice), 0·16 ml. of acetic anhydride was added and the samples were incubated for 2 hr in a water-bath at 60° C. Under these conditions aldosterone is transformed into its diacetate, whereas cortisol forms only a monoacetate, which is much more polar than the aldosterone diacetate. After acetylation the reagents were driven off *in vacuo*, care being taken that no traces were left behind.

Aldosterone, second chromatogram. The residues of the acetylation were dissolved in 0·3 ml. followed twice by 0·15 ml. of dichloromethane, and applied to paper. On each sheet there were two 8 cm lanes for the samples, and on each side a lane of 2·5 cm for the markers. The lanes were separated by 0·5 cm cut out strips. The samples were applied to the second 2 cm of the 8 cm starting line and run in a modified Bush B_3 system. The original B_3 system separates aldosterone diacetate and cortisol monoacetate well, but was sometimes found too fast for an over-night run. The speed of travel of the aldosterone diacetate was therefore decreased by changing the ratio of the solvents slightly in favour of the petroleum ether. The mixture finally used consisted of petroleum ether: benzene: methanol: water, 375:125:400:100 (v/v). In this system the spots of aldosterone diacetate are usually wide and large areas of the paper (8×9 cm) have to be eluted for quantitative recovery. This would cause high paper blanks if the final colorimetry were done on these eluates. Furthermore, small amounts of substances reacting with 'blue tetrazolium' were sometimes present close to the aldosterone diacetate region. Therefore the eluate of the aldosterone diacetate region was subjected to a third chromatographic procedure.

Aldosterone, third chromatogram. The eluates of the B_3 chromatogram were evaporated and the residues dissolved in 0·3 ml., followed twice by 0·15 ml. of dichloromethane and applied to washed papers (see below) as 1 cm^2 spots on 3 cm wide lanes which were separated by 0·5 cm cut out strips. Each sheet of paper consisted of seven such lanes; one at each end was used for the markers and two or three lanes served as paper blanks. Care was taken that the extracts did not reach the edges of the lanes. In order to reduce the blanks, papers used for this chromatogram were washed for 72 hr at room temperature. They were set up in tanks as for a descending chromatogram. For 24 hr the mobile phase of the modified A system (see below) was allowed to run over them. They were then transferred to a second tank where they were washed with a mixture of ethyl acetate-methanol 2:1 (v/v) for 48 hr. This is the mixture used for all elutions.

Bush's system A was used for the third chromatogram, but a little benzene was added in order to speed it up. The mixture thus consisted of petroleum ether (b.p. 80–100° C): benzene: methanol: water, 475:25:400:100 (v/v). During a 40 hr run in this system the aldosterone diacetate usually travelled 8–10 cm from the origin. The spots were discrete and not more than 20 cm^2 of paper had to be eluted. An additional advantage of running a third chromatogram is that the order in which the acetylated steroids run is not the same as in the

preceding system. In the B_3 system aldosterone diacetate runs a little faster than corticosterone monoacetate, whereas in the A system it runs more slowly. Though corticosterone monoacetate is not present in the extracts (corticosterone having been wholly removed in the first chromatogram), other impurities may behave in a similar way. We were, in fact, able to find occasionally an U.V.-absorbing substance contaminating the aldosterone region in the second chromatogram, but well separated from the aldosterone in the third. This substance did not react with blue tetrazolium.

Corticosterone and cortisol. Samples of up to 10 ml. of blood, plasma or cells of all species were extracted as described earlier for aldosterone. The residues of the final acetone extracts were applied to paper dissolved in 0·4 ml. dichloromethane. Whenever large samples of adrenal vein blood of the dog were worked up for aldosterone, one twentieth of the last ethanolic extract (see p. 138) was applied to paper for the estimation of cortisol and corticosterone. Each solution was applied in a 1 cm^2 area to 2 cm lanes, separated by $\frac{1}{2}$ cm cut out strips. Every sheet of paper had one or two blank lanes. On the two outermost lanes 10 μg control spots of cortisol and corticosterone were applied. A descending chromatogram was run in the solvent system, methanol:water:benzene, 275:225:500 (v/v) (B_5a; Bush & Sandberg, 1953).

Elution

A solvent mixture which is used to elute a substance from paper will be most efficient if the substance to be eluted travels with the front of the solvent mixture. Corticosteroids do this in a mixture of ethyl acetate-methanol 2:1 (v/v). This mixture was used for all elutions.

Aldosterone. The paper strips to be eluted from the aldosterone chromatograms were too large for the tubular eluters (see below). Therefore one edge of each paper strip was cut to a point. The other edge was inserted to a depth of 0·5–1 cm between two glass slides held together by a cotton or nylon thread. Rubber bands have to be avoided. The protruding portion of the paper strip was bent sharply over the edge of the slides, so that it hung vertically when the glass slides were stood in small glass troughs containing the eluting solvents. The eluate was collected in 10 ml. test tubes with ground-glass necks. The elution troughs and collecting tubes were fixed to stands which were placed in glass tanks. The walls of these tanks were lined with thick blotting paper which was soaked in the eluting mixture. The lid of the tank was sealed on with glycerine–starch paste. In order to achieve speedy and complete elution the tanks have to be saturated with the solvent. As the steroids travel with the front of the elution mixture, more than 80% are contained in the first drop of eluate. The elutions were usually allowed to run for 2 hr, when about 0·5 ml. of solvent had collected. Before putting the stopper on the elution tubes the necks had to be washed down carefully.

For recovery experiments quantities between 0·2 and 10 μg of different steroids were applied on 9×5 cm^2 paper strips and eluted. In thirty observations a mean recovery of 92% was obtained.

Corticosterone and cortisol. 6–7 cm long strips were cut out and eluted in small tubular glass eluters devised by Saffran & Sharman (1960).

Colorimetry

All steroids were estimated quantitatively by their colour reaction with blue tetrazolium in alkaline medium. The details for the determination of corticosterone and cortisol have been described previously (Vogt, 1955; Holzbauer & Vogt, 1957).

For the estimation of small amounts of aldosterone present in adrenal venous blood of the dog this method had to be modified by reducing the amount of some of the reagents. The dry residues of the final eluates were dissolved in 0·45 ml. of 95% ethanol (prepared to be free from aldehydes) and 0·04 ml. of a freshly made 1·25% solution of tetramethyl- or tetraethyl-ammonium hydroxide in 95% ethanol was added, followed by 0·05 ml. of a

freshly prepared 0·05% solution of blue tetrazolium (Gurr) in 95% ethanol. The solutions were well mixed, the tubes stoppered, and incubated in the dark for 1 hr at 23·5° C in a water-bath. The reaction was interrupted by adding 0·04 ml. of a 10% solution of acetic acid in 50% ethanol. The intensity of the developed colour was read against ethanol in the micro-cells of a Unicam spectrophotometer at a wave-length of 520 mμ. A standard solution was prepared from DL-aldosterone monoacetate (Ciba, ampoules of 1 mg/ml. 95% ethanol) by further acetylation to the diacetate. The contents of one ampoule were evaporated to dryness, taken up in 0·1 ml. of pyridine followed by 0·4 ml. of acetic anhydride and incubated at 60° C for 2 hr; the reagents were then driven off *in vacuo*. The residue was dissolved in 1 ml. absolute ethanol and stored at −16° C. The aldosterone diacetate in this solution ran as one spot in two different chromatographic systems.

A standard curve was calculated from thirty observations obtained on different days with amounts of aldosterone ranging from 0·5 to 8 μg. A straight line was obtained which ran through the origin and gave the conversion factor y/x of 0·073. The standard deviation from this calculated line is $\pm$0·018, which corresponds to $\pm$0·25 μg aldosterone monoacetate. Within one set of observations on any one day, however, amounts differing by 0·25 μg can be distinguished with confidence, as it is the whole set of estimations which may shift somewhat from day to day. For the special purpose of comparing different samples from one animal, the error is smallest if all estimations are done on the same day. The reagent blanks were equivalent to about 0·23 μg.

The samples which were eluted from the third chromatogram were read against paper blanks taken from other lanes of the same sheet of paper. Samples which had to be compared with each other were preferably run on the same sheet. The mean intensity of the colour reaction given by 51 paper blanks corresponded to 1·1 μg of aldosterone monoacetate with a standard deviation of $\pm$0·36 μg. This included the reagent blank. The blanks have recently been reduced to 0·8 μg by the use of glass-distilled water for the solvent mixtures. The mean difference between two blanks from two separate lanes of the same paper corresponded to 0·17 $\pm$ 0·17 μg of aldosterone monoacetate.

Identification

The identification of the aldosterone was limited to the R_F values in three different chromatographic systems and its sulphuric acid chromogen.

Recovery of steroids added to arterial blood

Aldosterone. For recovery experiments a sample of crystalline DL-aldosterone was dissolved in absolute ethanol and amounts containing 5 μg pipetted off into ampoules. These were freeze-dried, sealed and stored at −15° C. The following recovery experiments were carried out.

(1) Two samples of 5 μg aldosterone were acetylated and run in the modified B_3 and the modified A system. From both samples 4·5 μg were recovered.

(2) Two samples of 5 μg aldosterone were applied to paper, run in the E_2B system, acetylated and then run in the modified B_3 and in the modified A system. 4·5 and 4·1 μg were recovered.

(3) Blood was collected from the carotid artery of a dog under ether anaesthesia and kept in the refrigerator during the night. On the following morning it was well mixed and divided into six 100 ml. portions. Aldosterone 5 μg was added to two blood samples which were then extracted. Another two samples were first extracted and the aldosterone added to the unwashed ethylacetate extracts. The remaining two samples were used as blanks. No aldosterone was detected in these two samples. From the 5 μg aldosterone added to each of the other four samples 3 μg were recovered in each case. A similar experiment was carried out using three 100 ml. arterial blood samples of another dog. 5 μg aldosterone was added to two samples. The third was used as a control and no aldosterone was detected in it. Extrac-

tion, purification and chromatography of one sample were completed in 5 days. The sample was on paper for approximately 75 hr. 2·9 μg aldosterone was recovered. The final estimations for the other sample were carried out after 9 days, the extracts having been on paper for approximately 130 hr. Only 2·1 μg was recovered. Thus, losses of added aldosterone in the course of the chromatographic procedures are seen to be of the order of 15%, provided delays are avoided. 25% is lost in the preceding purification procedure. Evidence for losses of added aldosterone during the extraction was not obtained.

Cortisol and corticosterone. Recovery experiments for cortisol and corticosterone added to small amounts of plasma and extracts of adrenal tissue have been described previously (Vogt, 1955; Holzbauer, 1957). Two experiments were carried out in which 200 μg corticosterone and 200 μg cortisol were added to 200 ml. of dog's arterial blood. The samples were extracted and purified as described above and from each sample an aliquot of 1/20 was subjected to chromatography in Bush's system B_3a. 7·8 and 8·2 μg of cortisol and 8·2 and 10·3 μg of corticosterone were found; this is a mean recovery of 86%. The amounts of cortisol and corticosterone which can be expected in 1/20 of 200 ml. dog's arterial blood are below the threshold of the colorimetric method (< 1 μg).

Efficacy of the extraction as applied to adrenal venous blood

An experiment was carried out in which three different methods for the extraction of cortisol and corticosterone from a sample of dog's adrenal venous blood were compared. A 5 ml. portion was diluted with an equal volume of water and extracted three times with two volumes of the ethyl acetate-ether mixture. A second sample was diluted with three volumes of water and extracted three times with four volumes of the ethyl acetate-ether mixture. A third sample was diluted with one volume of water and extracted three times with two volumes of chloroform, shaking 100 times by hand as with ethyl acetate. The following estimates for cortisol (F) and corticosterone (B) were obtained:

Sample 1; 17·3 μg F and 9·2 μg B.
Sample 2; 18·3 μg F and 9·3 μg B.
Sample 3; 16·5 μg F and 8·5 μg B.

The chloroform extract was more difficult to handle because of the formation of emulsions. The results did not suggest that any of the modifications of the usual extraction procedure constituted an improvement.

Before analysis, the samples used for the experiment had been kept for 9 days at +4° C. Another sample of the same blood had been analysed (by the first method) on the day of collection. The amount of steroids found was 18·4 μg F and 9·3 μg B. Thus standing for 9 days at +4° C did not cause any appreciable loss of steroids.

In another experiment the possibility was tested that there was hydrogen bonding between steroids and blood proteins, which might prevent their extraction with organic solvents. To 87 ml. of adrenal venous blood of a dog 87 ml. of water was added, extraction was carried out as usual with three portions of ethyl acetate-ether, and aldosterone, cortisol and corticosterone were estimated in the extract. To the remaining blood-water mixture another 87 ml. of water was added, and the mixture acidified to pH 2 by the drop-wise addition of concentrated HCl with constant stirring. This mixture was extracted three times with 260 ml. ethyl acetate-ether and the combined extracts were prepared as usual for chromatography. The haemin formed by the addition of HCl was extracted with the organic solvents, but most of it was removed by washing the extracts twice instead of once with 1% Na_2CO_3. The bulk of the extract was used for the determination of aldosterone, and 1/20 for the estimation of cortisol and corticosterone. In the neutral extract 3·45 μg aldosterone were found, and in the acid extract an additional 0·4 μg aldosterone. This figure lies at the threshold of the method and is not very accurate. In 1/20 of the neutral extract 11·5 μg cortisol and 5·9 μg corticosterone were found. None (< 1 μg steroid) could be detected in 1/20 of the acid extract. However, in the E_2B chromatogram of the larger por-

tion (19/20) of the acid extract which was used for the estimation of aldosterone, there were U.V.-absorbing spots which had the R_F values of cortisol, cortisone and corticosterone. Their intensity corresponded to approximately 10 μg steroid or 5–10 % of the content of the neutral extract. From these results it was concluded that acidifying the blood-water mixture before extraction with organic solvents would, at best, increase the steroid yield by 5–10%. As it was more difficult to purify such extracts it was decided not to acidify and to accept the small loss.

RESULTS

Corticosteroids in the cell fraction of adrenal venous blood of the dog

Adrenal venous blood was collected from four dogs (50, 51, 54 and 57, Table 1) over different periods of time into tubes kept in ice-water. At the end of the collection the blood of each dog was divided into two portions.

TABLE 1. Extraction of steroids from fractions of adrenal venous blood of the dog. One volume of water only was added to each fraction

Dog no.	Collection time (min)	Total blood collected (ml.)	Volume of fractions extracted individually (ml.)		Steroid found (μg)			% of calculated* amount extracted from cells		
					Aldosterone	Cortisol	Corticosterone	Aldosterone	Cortisol	Corticosterone
50	70	560	Whole blood	280	6·8	304	224	—	—	—
			Plasma	132	1·2	194	124	—	—	—
			Cells	148	1·0	126	90	18	114	90
51	70	312	Whole blood	156	5·0	284	236	—	—	—
			Plasma	70	0·7	106	116	—	—	—
			Cells	86	1·2	98	84	28	55	70
54	90	202	Whole blood	101	3·5	382	244	—	—	—
			Plasma	48	0·9	184	138	—	—	—
			Cells	53	0·2	168	122	8	85	115
57	75	363	Whole blood	121	3·2	354	184	—	—	—
			Plasma	113	2·9	364	208	—	—	—
			Cells	129	2·3	364	104	66	106	65

The volumes extracted as whole blood are one half (dogs 50, 51 and 54) or one third (dog 57) of the total.

* The calculated amount is the difference between the quantity of a steroid found in whole blood and that found in plasma.

One portion (one half of the total in dogs 50, 51 and 54, one third in dog 57) was extracted as whole blood. The other portion was centrifuged for 15 min ($g = 2200$) and plasma and cells were extracted separately. To each sample an equal volume of water was added and estimation of aldosterone, corticosterone and cortisol carried out as described under Methods. The results (Table 1) show that in all four dogs the three steroids were extracted not only from the plasma but also from the cells. The amounts of aldosterone extracted from the samples of whole blood were up to three times larger than the amounts extracted from the same volume of blood after its separation into plasma and cells. In the estimates of cortisol and corticosterone this discrepancy was smaller or absent.

The next step was to try and determine the reason why, as just shown, aldosterone seemed to be more easily extracted from cells diluted with plasma than from packed cells. In the first experiment of Table 2 (dog 53), packed cells from adrenal blood were diluted with arterial plasma. Of the 370 ml. adrenal venous blood collected, one third was extracted as whole blood; two thirds were centrifuged and 106 ml. plasma and 140 ml. cells obtained. Each of these fractions was divided into two equal portions.

TABLE 2. Extraction of steroids from fractions of adrenal venous blood of the dog. Comparison of yield from packed cells lysed with one or with several volumes of water

Dog no.	Collection time (min)	Total blood collected (ml.)	Volume of fractions extracted individually (ml.)		Volume of water (or diluent) added	Steroid found (μg)			% of calculated* amou extracted from cells		
						Aldosterone	Cortisol	Corticosterone	Aldosterone	Cortisol	Cortic steron
53	135	369	Whole blood	123	1	5·5	148	88	—	—	—
			Plasma	53	1	3·0	68	64	—	—	—
			Plasma	53	1	2·9	70	56	—	—	—
			Cells	70	1	1·0	42	36†	39	53	128
			Cells	70	2·5‡	2·4	—	—	94	—	—
56	180	476	Whole blood	238	1	13·0	932	578	—	—	—
			Plasma	118	1	1·4	426	328	—	—	—
			Cells	60	1	3·4	118	100	59	47	80
			Cells	60	3	4·5	294	160	78	116	128
66	120	228	Whole blood	76	1	1·2	194	68	—	—	—
			Plasma	76	1	0·6	206	72	—	—	—
			Cells	76	3	1·8	214	72	100	117	112

The volumes extracted as whole blood are one half (dog 56) or one third (dogs 53 and 66) of the total.

* The calculated amount is the difference between the quantity of a steroid found in whole blood and that found in plasma. † values too near the threshold of the method to be accurate (glucocorticoids estimated in 1/20 of the whole extract). ‡ 53 ml. arterial plasma and 123 ml. water.

The whole blood sample (123 ml.), the two plasma samples (53 ml. each) and one cell sample (70 ml.) were extracted after the addition of equal volumes of water. To the second cell sample (70 ml.) 53 ml. of peripheral plasma of the same dog was added followed by 123 ml. of water. According to Table 2, $5{\cdot}5 - 3{\cdot}0\ \mu g = 2{\cdot}5\ \mu g$ aldosterone should have been found in the cell fraction, but only $1\ \mu g$ or 40 % of the calculated quantity was extracted from the packed cells. When, however, the cells were diluted with plasma and a larger volume of water before extraction, $2{\cdot}4\ \mu g$ or 94 % of the calculated value was found. No aldosterone ($< 0{\cdot}2\ \mu g$) was found in 53 ml. arterial plasma extracted separately.

In order to determine whether the beneficial effect of adding plasma to cells was simply that of suspending the cells in a larger volume of a watery phase, the experiments on dogs 56 and 66 were carried out. In dog 56 the collected blood was divided into two equal portions. One of these portions

was centrifuged and the cell fraction so obtained again divided into two halves. To the whole-blood portion, to the plasma separated from the cell fraction, and to one portion of the packed cells was each added one volume of water. Three volumes of water were added to the other portion of cells. The results (Table 2) show that suspending the cells in a larger volume of water increased to 78 % of the calculated value the amount of aldosterone that was extracted from the cells. In dog 66 one third of the blood collected was extracted as whole blood. The remaining two thirds were separated into plasma and cells. As before, one volume of water was added to the whole blood sample and to the plasma sample, and three volumes of water were added to the cell fraction. In this instance, the whole of the calculated amount of aldosterone was extracted from the packed cells.

Distribution of corticosteroids between cells and plasma in adrenal venous blood of dogs

Table 3 lists the concentrations of steroids found in plasma and cells of 15 samples of adrenal vein blood from eleven dogs. In some cases the concentration in the cells was calculated by difference from estimates in whole blood and in plasma. Figure 1 illustrates the ratios of concentration in plasma over concentration in cells for the three steroids. The ordinate is on a logarithmic scale. In samples collected for 75–135 min there is usually much more aldosterone in the cells than in the plasma. Cortisol is nearly equally distributed between the two phases. Corticosterone is always found in higher concentrations in the plasma though the excess in plasma is occasionally quite small. It can also be seen that the distribution of the three steroids between plasma and cells varies independently from dog to dog. There is also no consistent relation to the collection time.

Uptake of steroids by the cell fraction

Standing at +4° C

Aldosterone. This experiment was designed to test the time course of the uptake of aldosterone by the cells at +4° C. An adrenal blood sample of a dog was collected into polythene tubes immersed in ice-water. Each tube was placed into the centrifuge and spun exactly 20 min after the start of the collection. Within 2 hr 185 ml. plasma and 195 ml. blood cells had been collected. Two fifths of the plasma and two fifths of the cell sample were extracted immediately. The remaining three fifths of each sample were recombined and kept in the refrigerator (+4° C) for 120 min. The sample was shaken several times. Then it was spun and two thirds of the plasma and two thirds of the cell fraction were extracted separately. The remaining thirds of each fraction were re-combined and extracted as whole blood as a further check on the methods. The results are shown in Table 4.

After short contact between plasma and cells only about one third of the total aldosterone was found in the cell fraction. After a contact of more than 2 hr, over 70 % of the aldosterone had attached itself to the cells. There was no loss in the total amount of aldosterone during the time of the experiment.

In addition to the time during which cells and plasma are in contact, there must be other factors which influence the uptake of aldosterone by

TABLE 3. Distribution of steroids (μg/ml.) between plasma and cells in adrenal venous blood of the dog

Dog no.	Collection time (min)	Blood fraction	Aldosterone	Cortisol	Corticosterone
50	70	Plasma	0·009	1·47	0·94
		Cells	0·038	0·74	0·68
51	70	Plasma	0·009	1·52	1·66
		Cells	0·052	2·07	1·40
53	135	Plasma	0·055	1·30	1·13
		Cells	0·038	1·13	0·40
54	90	Plasma	0·019	3·84	2·88
		Cells	0·050	3·74	2·00
$55S_1$	90	Plasma	0·023	2·97	1·46
		Cells	0·035	2·28	1·40
$55S_3$	60	Plasma	0·057	—	—
		Cells	0·083	—	—
56	90	Plasma	0·012	3·61	2·78
		Cells	0·096	4·22	2·08
57	75	Plasma	0·026	3·22	1·84
		Cells	0·027	2·67	1·24
60	75	Plasma	0·026	1·01	0·93
		Cells	0·039	0·84	0·47
$60S_2$	75	Plasma	0·045	1·97	1·59
		Cells	0·090	1·82	0·42
61	75	Plasma	0·038	2·16	1·77
		Cells	0·035	2·25	1·50
$61S_2$	66	Plasma	0·114	5·32	3·78
		Cells	0·024	3·15	1·22
62	75	Plasma	0·057	3·93	2·79
		Cells	0·025	3·35	1·73
64	75	Plasma	0·012	4·00	2·91
		Cells	0·062	5·05	1·26
$64S_2$	75	Plasma	0·025	9·88	3·94
		Cells	0·154	6·58	1·07
Mean concentration		Plasma	0·035	3·30	2·17
		Cells	0·057	2·85	1·21
Mean ratio: $\frac{\text{concentration in plasma}}{\text{concentration in cells}}$			0·61	1·16	1·80

TABLE 4. Migration of aldosterone from plasma to cells in adrenal venous blood of the dog on standing for 2 hr at +4° C

Fraction	Time of contact between plasma and cells (min)	Volume (ml.)	Aldosterone (μg total)	Aldosterone (μg/ml.)	Concn. in plasma / Concn. in cells
Plasma	1–20	74	1·61	0·0218	2·09
Cells	1–20	78	0·81	0·0104	
Plasma	121–140	75	0·67	0·0089	0·40
Cells	121–140	76	1·67	0·0220	
Whole blood	—	76	1·24	—	—

the cells. This can be seen from Fig. 1. Sample 1 of dog 56 and sample 2 of dog 61 were centrifuged after approximately the same time of standing in ice-water. The cells of sample 56_1 contained the largest relative amount of aldosterone, those in sample 61_2 the smallest, ever observed in our experiments.

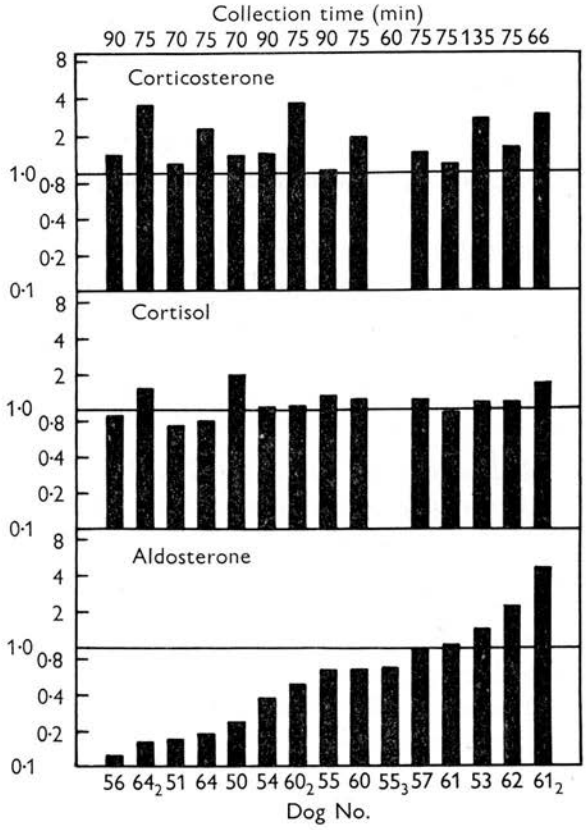

Fig. 1. Distribution of corticosteroids between plasma and cells of adrenal venous blood from the dog. Each bar represents the ratio (concentration in plasma): (concentration in cells) of a single sample. Different samples from the same dog are distinguished by the numeral below the dog number. Logarithmic ordinate scale.

Corticosterone and cortisol. Table 5 illustrates the corresponding experiment on the uptake by blood cells of corticosterone and cortisol. Dog adrenal venous blood was collected for 15 min into an ice-cooled polythene centrifuge tube and spun. Two fifths of the plasma and two fifths of the cell fraction were extracted immediately. The remaining three fifths of each sample were recombined, allowed to stand for 2 hr at +4° C, spun, two thirds of each fraction were extracted separately, and the two

TABLE 5. Cortisol and corticosterone in plasma and cells of adrenal venous blood of the dog before and after standing for 2 hr at +4° C

Fraction	Time of contact between cells and plasma (min)	Volume (ml.)	Cortisol (μg)	Corticosterone (μg)
Plasma	1–15	8·4	24·9	16·5
Cells	1–15	7·0	18·7	9·9
Plasma	121–135	8·4	21·5	15·7
Cells	121–135	7·0	22·5	11·6
Whole blood	—	7·7	20·1	13·2

remaining thirds combined and extracted together. The results are listed in Table 5. They show that the distribution of corticosterone and cortisol between plasma and cells has not significantly changed on standing.

In vivo distribution of cortisol and corticosterone between plasma and cells

Two sets of experiments were carried out in order to obtain information as to whether the distribution of the steroids between cells and plasma observed in the cooled, shed blood samples is also that existing *in vivo*, or is an artifact.

Collection at body temperature. It was possible that the entry of steroids into the cell fraction was favoured by cooling the blood as soon as it left the body. An experiment was carried out in which adrenal venous blood from a dog was collected over a period of 5 min in a polythene centrifuge tube, which was immersed in a water-bath of +37° C and gently shaken. The sample was spun immediately after the end of the collection. A second sample was then collected in a tube kept in ice-water and not shaken. It was also spun immediately after the 5 min collection period. The plasma and cell fractions were extracted separately and cortisol estimated. From the sample collected at +37° C, 24 μg cortisol was extracted from the plasma and 22 μg from the cells. From the sample collected at +2° C, 22 μg cortisol was extracted from the plasma and 23 μg from the cells. The short period of cooling had obviously not modified the distribution.

Collection in the presence of sodium fluoride. It was possible that glycolysis was involved in the association of steroids with blood cells. The inhibition of glycolysis during collection of the blood would then produce results different from those obtained with the usual collection procedure. Two 5-min samples of adrenal venous blood of a dog were collected in the following way. A polythene centrifuge tube containing one drop of a 0·65 % solution of NaF was immersed in a water-bath at +37° C and blood collection into it started. Throughout the collection 1 ml. of the NaF solution was added drop by drop to the blood in the tube, which was constantly shaken and then spun immediately. The final concentration of NaF in the

sample was 0·045 %, which is considered sufficient to inhibit glycolysis (Evans, 1922). A second sample was collected in the same way but 1 ml. of 0·9 % NaCl solution was added instead of the NaF solution. Corticosterone and cortisol were estimated in the cells and plasma of both samples. In the sample to which NaF was added 56 % of the total cortisol and 49 % of the total corticosterone were extracted from the cells. In the control sample 51 % of the cortisol and 46 % of the corticosterone were found in the cells. This result lends further support to the view that the association of corticosterone and cortisol with the blood cells takes place *in vivo*, immediately after their release from the adrenal cortical tissue.

Saline washing of blood cells

The stability of the association between cells and steroid was tested in two experiments. 40 ml. of dog's adrenal venous blood which had been standing for 3 hr at +4° C was spun. To a 5 ml. portion of the cells 15 ml. of saline was added and the mixture was shaken gently for 15 min at +21° C. It was then spun and the saline pipetted off. The cells were resuspended in another 15 ml. of saline, shaken for 15 min and spun. Corticosterone and cortisol were estimated in the washed cells and in each saline washing. These steroids were also estimated in 5 ml. untreated cells and 5 ml. plasma of the same blood. The extracting solvents were added to the samples after all of them had been kept at +21° C for a total of 90 min. This was done to allow any losses, attributable to the temperature at which the washing of the cells was carried out, to take place in the unwashed samples too. The experiment was repeated on another sample of dog's adrenal venous blood which had been kept for 6 days at +4° C (experiment 2). The results (Table 6) show that a large amount of the corticosterone and cortisol was washed off the cells by the saline.

TABLE 6. Removal of cortisol and corticosterone attached to cells of adrenal venous blood by washing with saline

Steroids extracted from	Experiment 1			Experiment 2		
	Volume (ml.)	Cortisol (μg)	Corticosterone (μg)	Volume (ml.)	Cortisol (μg)	Corticosterone (μg)
Plasma	5	18·0	8·4	4	12·6	10·2
Cells	5	14·5	6·1	4	10·9	4·8
Washed cells	5	4·8	2·7	4	2·3	0·7
1st saline washing	15	9·3	3·2	12	12·1	4·0

Second saline washings: steroids were also detected in the second saline washings. The amounts were, however, near the threshold of the method and the values are therefore not listed in the table.

Presence of steroids in cells of arterial blood of the dog

Blood was collected from the carotid artery of a dog under ether anaesthesia. It was centrifuged. 250 ml. of the cells was diluted with 750 ml. of water and extracted. The extract was tested for corticosterone, cortisol and aldosterone. 8 μg corticosterone and 12 μg cortisol were found. There was an U.V.-absorbing spot visible in the region of aldosterone diacetate on the third chromatogram, but the eluate of this region did not react with blue tetrazolium. The concentration of cortisol in the cells was of the same order as the average concentration of cortisol in the plasma of blood from the carotid artery: in eight dogs there was a mean of 9·8 μg cortisol in 250 ml. plasma (range: 5·7–12·1 μg).

Distribution of steroids between plasma and cells of adrenal venous blood from rabbit, rat and guinea-pig

Adrenal venous blood samples were taken from a rabbit, a rat and a guinea-pig. The blood was collected into siliconed glass tubes kept in ice-water, spun, and the glucocorticoids were estimated in the plasma and cells. The results are shown in Table 7. In these three species the distribution of the glucocorticoids between plasma and blood cells was similar to that in the dog, about 75 % of the corticosterone being found in the plasma, and the cortisol being distributed equally.

TABLE 7. Distribution of corticosteroids between plasma and cells in adrenal venous blood of rabbit, rat and guinea-pig

Species	Collection time (min)	Fraction	Volume (ml.)	Steroid	(μg total)	(μg/ml.)	Concn. plasma / Concn. cells
Rabbit	20	Plasma	11	Corticosterone	9·0	0·82	3·28
		Cells	10		2·5	0·25	
Rat	35	Plasma	2·5	Corticosterone	17·6	7·04	2·42
		Cells	2·2		6·4	2·91	
Guinea-pig	20	Plasma	2·0	Cortisol	12·6	6·30	0·95
		Cells	2·5		16·6	6·64	

Comparison of values for the secretion rate of corticosteroids, calculated from estimates in plasma and in whole blood

Basic secretion rate in dogs. 'Control' samples of adrenal venous blood were collected from dogs at the beginning of ten experiments, the lost blood being replaced by slow infusion of donor blood. Each sample was divided into two parts. One part was extracted as whole blood, the other centrifuged and the plasma extracted. For each dog the secretion rate of aldosterone, corticosterone and cortisol by the left adrenal was calculated, based on the estimations in plasma and in whole blood and expressed as

μg/adrenal/hr/kg body weight. The mean, the standard deviation of the mean and the coefficient of variation were calculated for each group of figures (Table 8). As was to be expected from the findings on the distribution of aldosterone between plasma and cells, the mean values calculated on the basis of whole-blood estimations are more than three times as large as those calculated from the plasma content only. Furthermore, the coefficient of variation of the secretion rate of aldosterone between individual dogs is smaller when the figures are based on values in whole blood. This agrees with the previous finding that there are large individual variations in the association of aldosterone with blood cells. In contrast, the secretion

TABLE 8. Calculation of secretion rates based on concentrations of steroids (*a*) in plasma and (*b*) in whole blood (μg steroid secreted/hr and kg body weight by the left adrenal)

Dog no.	Collection time (min)	Aldosterone		Cortisol		Corticosterone	
		Plasma	Whole blood	Plasma	Whole blood	Plasma	Whole blood
50	70	0·12	0·68	19·5	32·3	12·4	22·7
51*	70	0·08	0·58	12·1	32·7	13·3	27·1
53	135	0·31	0·57	6·8	13·2	5·9	8·7
54†	90	0·08	0·33	22·8	35·3	12·8	17·0
55	90	0·18	0·51	22·6	44·5	11·1	24·8
56*	90	0·10	0·66	24·7	52·4	21·1	35·3
57	75	0·23	0·50	30·2	53·3	16·3	28·9
60	75	0·31	0·89	10·8	23·0	10·1	16·8
61	75	0·28	0·55	15·8	33·4	12·9	23·5
64	75	0·06	0·38	19·8	46·0	14·5	21·0
Mean		0·175	0·565	18·5	36·6	13·0	22·6
Standard deviation		0·1	0·16	7·1	12·7	3·9	7·3
Coefficient of variation (%)		57	28	38	35	30	32

* Splanchnic nerves cut on left side; † splanchnic nerves cut on both sides.

rate of cortisol and corticosterone, when based on concentrations in blood, is only about twice that based on plasma concentration, reflecting the fact that approximately equal amounts of these steroids are found in plasma and in cells. Individual variations are not greater when plasma is used as the basis for the calculation. This is due to the greater constancy of the distribution of corticosterone and cortisol between plasma and cells.

Estimation of changes in the secretion rate of steroids occurring during the collection of consecutive blood samples from the same dog

Experiments were carried out in order to investigate whether, within an experiment on one dog, changes observed in the secretion rate of adrenal steroids were the same when calculation was based on the steroid content of plasma or that of whole blood. Two samples were collected from each of five dogs. In three dogs the splanchnic nerves were cut before collecting

the second sample, in one dog the second sample was taken 1 hr after a severe haemorrhage, when the adrenal blood flow was very slow. In these four experiments (Table 9) each sample was divided into two portions, one portion was spun and the plasma alone extracted, the other portion was extracted as whole blood. The percentage change in the secretion rate of aldosterone was then calculated from estimates in the plasma fraction only and from estimates in whole blood. In addition, Table 9 contains the percentage of the total amount of aldosterone present in the plasma fraction. In dogs 55, 60 and 64 any alterations in aldosterone secretion occurring in whole blood were reflected in the plasma content. In these dogs the

TABLE 9. Secretion rates of aldosterone during the collection of two consecutive samples, calculated from estimations in plasma and from estimations in whole blood

Dog no.	Sample no.	Treatment between collection of samples	Time of collection (min)	Adrenal blood collected (ml.)	Homologous blood infused (ml.)	Haematocrit (% red cells)	Aldosterone in plasma (content of blood = 100)	% change secretion rate betwee samples wh estimated in
55	1	Bled	90	455	450	56	34·7	Blood −
	3		60	88	273	56	35·2	Plasma −
60	1	Splanchnotomy	75	370	341	57	32·1	Blood +
	2		75	210	375	55	29·2	Plasma +
61	1	Splanchnotomy	75	360	280	46	50·0	Blood −
	2		66	142	600	55	79·5	Plasma +
64	1	Splanchnotomy	75	166	150	51	15·9	Blood +
	2		75	88	175	47	15·8	Plasma +

percentage of the total aldosterone found in plasma was the same in both samples. In dog 61 an interesting discrepancy arose: after splanchnotomy, it was difficult to keep up the blood pressure by infusing donor blood, and 600 ml. of donor blood had to be infused within 1 hr, whilst only 142 ml. blood was collected from the adrenal vein. Aldosterone secretion, calculated on the basis of the concentration in blood, was slightly lower in the second sample. When, however, calculation was based on the values in the plasma, secretion rate appeared to have risen. This was due to the fact that the distribution of aldosterone between plasma and cells had shifted in favour of the plasma during the course of the experiment, a shift probably caused by the infusion of excessive amounts of foreign blood. In Table 3 it was shown that the distribution of aldosterone between plasma and cells is subject to large individual variations, and the infusion of a large volume of blood from another dog may, therefore, lead to a serious alteration in the relative amount of aldosterone in cells and plasma.

In a fifth dog (131, Table 10) the effect on the distribution of steroids between cells and plasma of infusing a large quantity of dextran was tested. 300 ml. of Dextraven (dextran 6 g/100 ml., in glucose solution, 5 g/100 ml.,

salt-free, Benger Laboratories Ltd.) were infused between the collection of sample one and sample two, and another 350 ml. Dextraven during the collection of sample two. Both samples were collected over a period of 45 min into ice-cooled polythene tubes and the samples were centrifuged. Cells and plasma were not in contact with each other for more than 30 min. Before extraction one volume of water was added to the plasma samples and three volumes to the cell samples. When calculation of the secretion rate of aldosterone was based on estimations in plasma, it appeared to have

TABLE 10. Changes in the distribution of corticosteroids between plasma and cells in two 45-min samples of adrenal vein blood (Dog 131). After collection of sample 1 infusion of 300 ml. dextran dissolved in glucose

2	3	4	5	6	7		8	9	10	11	12	13
			Concentration in plasma				Secretion rate of left adrenal (μg/hr/kg b.w.)					
Adrenal vein blood collected (ml.)	Haematocrit (%)	Homologous blood infused (ml.)	Concentration in cells				Aldosterone		Cortisol		Corticosterone	
			Aldosterone	Cortisol	Corticosterone		μg	% change	μg	% change	μg	% change
330	64	100	1·19	0·95	1·21	Plasma	0·20	—	17·5	—	12·3	—
						Whole blood*	0·48	—	49·8	—	29·9	—
270	37	75, + 350 dextran	0·61	1·61	1·44	Plasma	0·25	+25	27·9	+59	16·1	+31
						Whole blood*	0·49	−0·6	38·1	−23	22·7	−24

* Sum of quantities estimated separately in cells and plasma.

risen in sample two. However, when one adds the amount found in plasma to that found in the cells it is obvious that there was no rise. Further scrutiny shows that it is only by chance that the discrepancy between these two methods of assessment is not greater still. The infusion of dextran brought about two changes which had opposing effects. First, it caused a decrease in the haematocrit of the second sample, which would tend to increase the fraction of the total aldosterone present in the plasma. Second, it caused an increase in the association of aldosterone with the blood cells; this, on its own, would decrease the fraction of the total aldosterone present in the plasma.

The discrepancies were larger still when the changes in the secretion rates of cortisol and corticosterone were calculated from estimates in plasma and compared with those calculated for whole blood. Whereas the dextran caused a shift of the aldosterone to the cells, it altered the distribution of the cortisol in favour of the plasma (column 6, Table 10). That shift, together with the larger plasma volume of sample two, caused a rise in the amount of cortisol in the plasma of sample two. This, however, did not represent a rise in secretion, because there was a very large fall of the cortisol content of the cell fraction. Calculated for whole blood the real change in secretion rate was a fall of 23 %.

Plasma estimations of corticosterone also gave a wrong impression of the change in the secretion rate during collection of sample two (last column, Table 10), but the discrepancy was less than for cortisol owing to the smaller change in the distribution coefficient of corticosterone between plasma and cells (column 7, Table 10).

From the results of these experiments it follows that estimates of steroids in two samples of plasma will only reflect changes in the secretion rate of the steroids when the haematocrit and the distribution coefficient of the steroid between plasma and cells remain constant. Both these factors can change during an experiment if large amounts of foreign blood or dextran are infused. Correction of plasma estimates for changes in the haematocrit serves no useful purpose unless it is known that the distribution of the steroid between plasma and cells has remained the same.

DISCUSSION

The experiments have demonstrated the presence of corticosteroids in the cell fraction of adrenal venous blood in four different species. This fact has not been fully appreciated by other workers, possibly because of the difficulty in extracting steroids from the blood cells. There is little doubt that the steroids extracted from the cells are chemically identical with those extracted from the plasma.

The association of steroids with the cells is easily destroyed by washing the cells with saline. This suggests that the cell-bound steroids are available for use by the tissues. The low concentration in the cells of carotid arterial blood supports this view.

We have no evidence on the type of cell with which the steroids are associated. The cell fraction in these experiments consisted of red cells, white cells and some platelets. There is also no conclusive evidence on the site of this association, whether it be intracellular or at the surfaces of the cells, though the easy removal by washing or during circulation in the vessels suggests an attachment to the surface. The volume of plasma trapped between packed cells is too small to account for the large amount of steroids found.

The possibility that the presence of steroids in association with the cells arises as an artifact during the collection of the blood was investigated for cortisol. Quick collection of blood maintained at 37° C and followed by as rapid a separation as possible of the cells from the plasma, did not reduce the amount of cortisol extractable from the cells; this was so whether or not glycolysis was inhibited during collection. It therefore appears that association of glucocorticoids with the cells occurs to a large extent *in vivo*.

On the other hand, experiments carried out on the uptake of aldosterone

when blood was stored at $+4^{\circ}$ C suggested that *in vivo* less aldosterone is associated with the cells than was found by our method of collecting the blood over a long period of time before separating the cells from the plasma. Whereas during the time taken for collection there is a migration of aldosterone from plasma to the cells, no such shift was observed with cortisol and corticosterone.

The possibility that the cell fraction of the blood acts as a carrier mechanism of hormones *in vivo* has to be seriously considered. Binding to blood cells *in vitro* also occurs with other hormones. Cohen (1959) has reported that more than 70 % of adrenaline and noradrenaline added in small quantities to peripheral human blood can be taken up by blood cells. The kinetics of the association of hormones with blood cells are so far unknown.

Previously estimations of the secretory activity of the adrenal cortex were often based on observations made on plasma extracts alone. The experiments described here suggest that using this method of estimation may lead to errors. Secretory changes indicated by estimation of corticoids in plasma are only reliable if the haematocrit and the degree of association of the steroid with the blood cells is the same in both samples. Changes of these two factors were observed after infusion of large amounts of homologous blood or of dextran.

SUMMARY

1. Corticosteroids were extracted from the cells of adrenal venous blood of four mammalian species.

2. In 15 samples of adrenal venous blood of dogs collected over 60–120 min the ratio of concentrations [plasma aldosterone]:[cell aldosterone] varied from 0·125 to 4·7, with a mean of 0·61. Cortisol was found in approximately equal amounts in cells and plasma. The concentration of corticosterone was usually higher, and never lower, in the plasma than in the cells.

3. The steroids could be removed from the cells by washing with saline. Only small amounts of cortisol and corticosterone were found in the cell fraction of blood from the carotid artery of a dog, the concentration being of the same order as that of the plasma. No aldosterone ($< 0{\cdot}2$ μg) was detected in as much as 250 ml. of packed red cells from carotid blood.

4. The proportion of corticosteroids, particularly of aldosterone, associated with the cells in adrenal vein blood is so variable that it is preferable to estimate these steroids in whole blood rather than in plasma when the secretion rate of these hormones is under investigation. Extraction of steroids from red cells presents no difficulties if enough water is added to the packed cells before extracting with the organic solvent.

We wish to thank Miss Margaret Crookston and Mr David Flockhart for their careful technical assistance. We are grateful to Mr N. E. Condon for designing and making the rotating clamp used for *in vacuo* evaporations. Benger Laboratories Ltd. have kindly supplied us with sodium-free 'Dextraven' and Ciba Laboratories Ltd. with samples of aldosterone.

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INVESTIGATIONS INTO THE CAUSES OF THE RISE IN ALDOSTERONE SECRETION DURING HAEMORRHAGE

PARTS I AND II

By

MARGARETHE HOLZBAUER AND MARTHE VOGT, F.R.S.

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INVESTIGATIONS INTO THE CAUSES OF THE RISE IN ALDOSTERONE SECRETION DURING HAEMORRHAGE. PART I

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(*Received* 31 *August* 1965)

CONTENTS

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The effect of haemorrhage on aldosterone secretion was studied in anaesthetized dogs with intact pituitary glands and kidneys subjected to the stress of adrenal vein cannulation. The following observations were made:

Acute haemorrhage was followed by a significant rise in aldosterone secretion in about one half of the animals studied.

In most of the remaining dogs, called non-reactors, premature stimulation of aldosterone secretion before the withdrawal of blood appeared to be the cause for the lack of response. This stimulation was traced in many instances to prolonged surgical 'stress', in others to incipient circulatory failure.

Another reason for a high initial secretion rate of aldosterone was low dietary sodium intake continued for a week or more.

Increase in aldosterone secretion after haemorrhage was unimpaired by sectioning the vagi or the splanchnic nerves, and by the absence of the proprioceptors of carotid sinus and thyro-carotid junction, or of liver, spleen and gastrointestinal tract.

During haemorrhage there is secretion of medullary amines and anoxia develops. The effect of these factors on aldosterone secretion was tested by infusing adrenaline and noradrenaline in the splanchnotomized animal, and by carrying out exchange transfusions with plasma till the dog had lost 50 % of its red cells. Provided the initial aldosterone secretion was low enough, these procedures caused small rises in output of aldosterone, but constituted less effective stimuli than blood loss.

Glucocorticoid secretion was in all animals maximal or near maximal and changed but little in the course of the experiments.

The findings suggest that, in the intact dog, aldosterone secretion is influenced by a variety of factors, most of which act indirectly by releasing *ACTH*, or renin, or both. The role of *ACTH* and of renin as mediators of the action of haemorrhage on secretion of aldosterone will be studied in part II.

INTRODUCTION

Acute haemorrhage stimulates aldosterone secretion in the dog (Farrell, Rosnagle & Rauschkolb 1956). This observation has been confirmed, extended to other species, and used as a standard stimulus in investigations on the control of aldosterone secretion in several laboratories. The present and the following paper deal with experiments which were designed to study the mechanism of this phenomenon.

Sudden withdrawal of substantial amounts of blood calls into action a great number of compensatory mechanisms, most of which are concerned with the maintenance of the arterial pressure at a level sufficient to insure adequate oxygen supply to the vital organs. The mechanism best studied is the increase in sympathetic activity (Cannon 1932) which results in vasoconstriction in the splanchnic bed and certain muscle areas and is triggered by baroreceptors of the carotid sinus and the aorta (Heymans & Neil 1958). Closely related are the effects caused by the catecholamines released from the adrenal medulla (Bedford & Jackson 1916). A different mechanism which contributes towards maintaining the blood

pressure is the release of renin during bleeding (Huidobro & Braun-Menendez 1942; Scornik & Paladini 1964). Furthermore, haemorrhage causes release of *ACTH* (Sydnor & Sayers 1954) and of vasopressin (Ginsburg & Heller 1953; Ginsburg 1954; Weinstein, Berne & Sachs 1960). These factors have to be considered in an analysis of the processes involved in the rise in aldosterone secretion following haemorrhage.

All experiments described in this part of the work were carried out under conditions of operative stress in the presence of the pituitary gland and the kidneys, conditions under which both aldosterone and glucocorticoid secretion were already elevated prior to haemorrhage. Therefore it was necessary to determine how consistently, under these circumstances, a rise in aldosterone secretion would follow haemorrhage. Then experiments were designed to test whether receptors in any particular organ might be found to initiate the response, whether it disappeared in the absence of the vascular beds of liver and gastrointestinal tract, of the nerve supply to the baroreceptors and chemoreceptors of the carotid arteries, of the splanchnic nerves or of the vagi. Furthermore a possible participation of adrenal medullary hormones was studied. Finally, tissue oxygen tension was lowered in order to test whether chemoreceptor stimulation could lead to a rise in aldosterone secretion.

METHODS

Operative procedures

Mongrel dogs of both sexes were used and numbered in chronological order. Dogs 15 to 113 were used 1 to 4 days after admission so that their previous electrolyte intake was essentially unknown. The dogs of the subsequent experiments were often kept for long periods. Dogs 114 to 158 had a daily Na^+ intake in the range of only 30 m-equiv. (milli-equivalents). Later the food was supplemented by 70 m-equiv. Na^+/day in the form of NaCl so that the daily sodium intake was about 100 m-equiv. The potassium intake was about 65 m-equiv. throughout. Starting with dog 239, all dogs were vaccinated against distemper on admission.

The anaesthesia used was ether followed by chloralose (70 to 80 mg/kg, 0·7 to 1·0% solution in 0·9 % sodium chloride, i.v.). When required, artificial respiration was given through a tracheal cannula by a Starling pump, 20 rev/min, stroke 15 ml./kg body weight. One adreno-lumbar vein was approached either by an abdominal midline incision or an incision in one flank and prepared for collection of adrenal blood in such a way that the blood could be shunted into the femoral vein between periods of collection. Dogs 236 to 339 received an intravenous infusion of suxamethonium bromide B.P., 0·05 % solution in 0·9 % sodium chloride, about 0·3 ml./min, while the abdomen was opened and the adreno-lumbar vein dissected. Heparin, 350 to 450 u./kg, was injected before adrenal vein cannulation. Femoral arterial blood pressure was recorded with a mercury manometer. Blood losses incurred during the collection of adrenal venous blood and by spontaneous bleeding were replaced by infusions of donor blood. This was obtained from dogs which were either freshly admitted or had been kept on the same diet as the experimental dogs. The blood was preserved as described previously (Holzbauer 1964). Mepyramine maleate (5 mg/kg to dogs 49 to 217, 2 mg/kg to dogs 218 to 235, 1 mg/kg to all further dogs) was given before infusing donor blood in order to try and minimize sensitivity reactions which often result from intolerance to homologous blood (Bliss, Johns & Burgen 1959; Remington & Baker 1959). Starting with

dog 272 an approximate estimate of the amount of blood lost by wound bleeding was obtained by covering the wounds with weighed pads of cotton wool. Adrenal blood flow as given in tables and figures represents all the blood collected per hour from the cannulated adreno-lumbar vein after ligation of accessible tributaries. Small blood specimens for the estimation of the haematocrit and the Na^+ and K^+ content of the plasma were taken from a femoral artery.

Variations in the operative procedures as they were necessitated by specific problems are discussed in the corresponding sections.

Chemical procedures

The methods used for the extraction of steroids from adrenal venous plasma or blood, the purification of the extracts, separation of the individual steroids by paper chromatography in three different systems and their quantitative estimation by a colour reaction with blue tetrazolium have been developed in the course of this investigation and differ slightly at different stages of the work. Detailed descriptions of the chemical methods as they were used at an early and at the last stage of the investigation have been given previously (Holzbauer & Vogt 1961; Holzbauer 1964).

Essential modifications introduced in the course of the work were as follows:

Extraction

The adrenal blood samples of dogs 15 to 54 were spun immediately after collection and only the plasma was extracted. In all further experiments whole blood was analysed.

Purification of extracts

Starting with the samples from dog 138, the ethanolic phase obtained after defatting the extracts, and the acetone remaining after precipitation of the phospholipids, were no longer evaporated *in vacuo*, but taken to dryness in a water bath of 40 to 50 °C by means of a stream of N_2. This was replaced later by a stream of filtered compressed air.

Chromatography

The width of paper used for chromatography was gradually decreased without loss of efficiency, the final sizes being those described by Holzbauer (1964).

Elution

The time allowed for the elution of larger strips of paper was extended from 2 h to 3 to 4 h starting with dog 236; with the shorter elution period there might have been occasional losses.

Colorimetry

All steroids were estimated by the colour formed with blue tetrazolium in an alkaline medium. The method described by Vogt (1955) was used for the estimation of cortisol and corticosterone throughout the work, with the exception of the experiments on hypophysectomized dogs, in which a micromethod (Holzbauer & Vogt 1961) was employed. This micromethod was used throughout for aldosterone starting with experiment 41.

'Paper blanks'

In order to remove non-steroidal impurities which are eluted from chromatography paper and which reduce blue tetrazolium, the papers used for the last chromatogram of aldosterone were washed. The procedure always included 48 h washing with a mixture of ethylacetate:methanol, 2:1. This was later found to be the only essential part of the washing procedure. It was also used for the chromatography of cortisol and corticosterone when small amounts of steroid only were available as in samples from hypophysectomized dogs. Eluates from one to three blank areas of each final chromatogram were reacted with blue tetrazolium and their photometer readings ('paper blank') subtracted from the reading of the eluate containing the steroid. At different periods of the work these paper blanks, expressed in μg aldosterone, varied; in any one group of estimations carried out on any one day a difference in aldosterone values between two extracts was only considered to differ from zero if it exceeded twice the highest difference between blanks. From dog 184 onwards redistilled benzene and petroleum ether were used for the last chromatogram. This reduced the blanks to a mean which was equivalent to 0·35 μg aldosterone, and the variation between blanks became very small.

Recoveries

The results of recovery experiments carried out by adding to peripheral blood non-radioactive aldosterone, cortisol and corticosterone in quantities likely to occur in the samples of adrenal venous blood have been described (Holzbauer & Vogt 1961). When radioactive aldosterone became available, it was possible to measure the losses of aldosterone sustained in each individual sample. Beginning with dog 232, a known quantity of 7-^{3}H-aldosterone was added to the blood immediately after collection, and the recovered radioactivity was measured after having carried out the colour reaction between aldosterone and blue tetrazolium. Originally, each coloured solution was transferred to a counting vial, evaporated to dryness and the scintillator was added. In this way no quenching occurred. It was, however, found that evaporation to dryness in the presence of the chemicals used for the reaction with blue tetrazolium sometimes led to losses in counts of up to 10 %, independent of the absolute amount of counts present. Starting with dog 284, the method was therefore changed to that already published (Holzbauer 1964) in which evaporation was avoided and the degree of quenching due to the ethanol estimated and taken into account. In all experiments in which radioactive aldosterone was used, the figures obtained from the reaction with blue tetrazolium were corrected for 100 % recovery. The validity of this calculation was investigated in experiments in which unlabelled and radioactive aldosterone were added to samples of peripheral blood and the samples were carried through the full extraction and estimation procedures. When the estimates for aldosterone obtained with the colour reaction were corrected for the losses estimated by counting the radioactivity, the mean amount of aldosterone found was 117 % $\pm$ 3·4 % (S.E.) of the amount added. It was, however, observed that small amounts of a substance reacting with blue tetrazolium are present in the aldosterone diacetate region of the last chromatogram of 'blank' samples of peripheral blood. If this quantity is subtracted from the result the figure for the mean corrected recovery of aldosterone becomes 103 % $\pm$ 3·4 % (S.E.).

At a later stage of the work a decrease of the specific activity in the sample of 7-^{3}H-aldosterone was observed. Only about 65 % of the counts were found in the aldosterone region of paper chromatograms developed in Bush's system B_5. Most of the remaining activity had travelled with the solvent front. Allowance for this disintegration was made when losses were calculated.

From results obtained on 410 samples a mean recovery of 33 % (S.D. $\pm$2·4) of the aldosterone added at the beginning of the extraction procedure was calculated. The amount of radioactivity recovered was independent of the size of the original blood sample (30 to 200 ml.) and was the same in samples of whole blood, plasma or blood cells.

Estimation of the plasma concentration of Na^+ *and* K^+

Sodium and potassium were measured in the plasma of samples from arterial blood with an 'EEL' flame photometer.

RESULTS

I. Effect of haemorrhage on aldosterone secretion in the anaesthetized dog subjected to adrenal vein cannulation only

Procedures and observations

Variability of basal cortical secretion rates during consecutive periods of collection

In eight dogs the variability of corticoid secretion was studied by analysing two or three samples of adrenal blood collected at short intervals. The adrenal vein was approached through a midline incision or from the flank and collection was begun 30 to 60 min after completion of the dissection, collection periods being 20 to 25 min with intervals of 15 min.

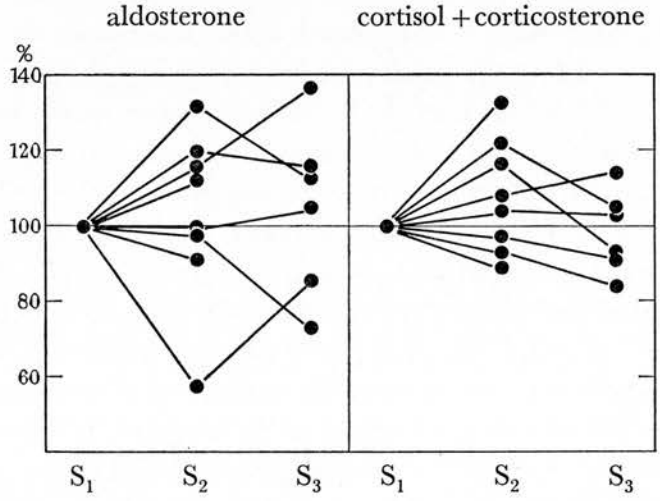

Figure 1. Spontaneous changes in adrenal steroid secretion during three consecutive collection periods of 20 to 25 min (S_1, S_2, S_3); 15 min intervals between collections. Results expressed as percentage of initial secretion rate.

The results are shown in figure 1. The largest rise in aldosterone secretion observed in two consecutive periods was 30 % in one dog. Changes in glucocorticoid secretion rates also reached 30 % only once. From these observations it was assumed that rises in aldosterone

secretion above 30 % do not occur under constant experimental conditions. All dogs which showed rises of more than 30 % in response to haemorrhage will therefore be referred to as 'reactors', those which failed to do so as 'non-reactors'.

Aldosterone secretion following expansion and subsequent reduction of the blood volume

These were early experiments in which corticoids were estimated in plasma. The adrenal vein was cannulated from a midline incision.

In a first series of dogs the blood volume was expanded by dog's plasma or by plasma substitutes (dextran or polyvinylpyrrolidine) and then reduced by bleeding. Significant changes in aldosterone secretion were only obtained after reduction of the blood volume, but interpretation was complicated because a number of dogs bled profusely as a result of the infusions; furthermore, the plasma substitutes were often poorly tolerated.

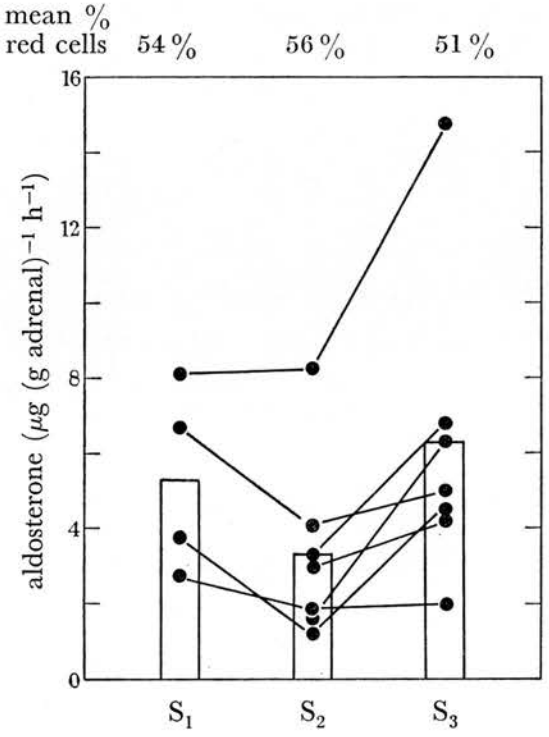

FIGURE 2. Effect of expanding and reducing the blood volume on the secretion rate of aldosterone (μg (g adrenal)$^{-1}$ h^{-1}). S_1, before blood infusion (mean and S.E. $5{\cdot}3 \pm 1{\cdot}1$); S_2, after infusion of blood, 20 ml./kg (mean $3{\cdot}3 \pm 1{\cdot}0$); S_3, after haemorrhage (mean $6{\cdot}3 \pm 1{\cdot}6$). Salt intake not controlled. First sample taken immediately after completion of operation. (Steroids extracted from plasma; aldosterone figures not corrected for losses; ●, individual figures; columns, means).

In a second series dog's blood was used to expand the blood volume before taking sample marked S_2 (figure 2), and also to replace the blood losses incurred during collection of adrenal blood. Variable and often very severe bleeding from the wounds followed hypervolaemia produced by homologous blood. Therefore, the amount of blood which could be safely withdrawn from the femoral artery before collection of sample 3 without causing shock was occasionally negligible (3 ml./kg) and sometimes as high as 30 ml./kg. Figure 2 shows that five of seven dogs reacted to blood loss with increases in aldosterone secretion

ranging from 40 to 318 %, some of these changes being larger than the falls following expansion. In view of the excessive bleeding caused by infusions of large volumes of homologous blood, experiments using expansion of the blood volume were discontinued and haemorrhage alone was used as the standard stimulus for aldosterone secretion.

Aldosterone secretion following haemorrhage; 30 min interval after surgery

In these and all subsequent experiments, corticoids were extracted from whole blood. In a first group of 15 dogs (tables 1 and 2), adrenal blood collection was started 30 min after completion of dissection.

TABLE 1. EFFECT OF HAEMORRHAGE ON ALDOSTERONE SECRETION

First adrenal blood sample (S_1) collected 30 min after end of dissection; second sample (S_2) after haemorrhage. Dietary sodium intake not controlled, midline incision. (Steroids extracted from whole blood, aldosterone figures not corrected for losses.)

dog no.	body wt. (kg)	adrenal blood sample no.	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change) aldosterone	cortisol	corticosterone	adrenal blood flow (ml./h)	mean b.p. (mmHg)	Mayer waves	blood withdrawn between S_1 and S_2 (ml./kg)
			Group I. Reactors						
108, male	13·5	S_1	6·1	521	230	276	122	−	24
		S_2	9·2 +51	744 +43	230 0	161	74	+	
109, female	21·6	S_1	0·8	372	309	600	130	−	25
		S_2	3·0 +275	491 +32	261 −16	216	52	−	
111, male	17·1	S_1	4·7	972	605	336	138	−	28
		S_2	8·1 +72	1093 +12	460 −33	143	60	+	
mean ±s.e.		S_1	3·9 ± 1·6	622 ± 180	381 ± 114	404 ± 100	130 ± 4·6	·	26 ± 1·2
		S_2	6·8 ± 1·9	776 ± 175	317 ± 72	173 ± 22	62 ± 6·4		
			Group II. Non-reactors						
55, male	17·0	S_1	11·3	983	547	300	135	+	22
		S_2	9·2 −19	769 −22	275 −50	140	64	++	
99,* female	17·1	S_1	4·7	655	338	624	160	−	23
		S_2	5·8 +23	753 +15	305 −10	396	80	+	
102,* male	21·6	S_1	14·0	1299	558	272	160	−	14
		S_2	11·9 −15	1072 −18	346 −38	168	100	+++	
107,† male	16·6	S_1	8·3	747	362	459	128	−	22
		S_2	8·3 0	894 +20	272 −25	150	55	−	
mean ±s.e.		S_1	9·6 ± 2·0	921 ± 144	451 ± 59	414 ± 81	146 ± 8·4	·	20 ± 2·1
		S_2	8·8 ± 1·3	872 ± 74	299 ± 17	214 ± 61	75 ± 9·9		

* Some blood withdrawn before S_1. † Cheyne Stokes respiration.

In the seven dogs of table 1 salt supplements were not given, and the aldosterone figures were not corrected for losses.

Table 1 shows that the mean initial aldosterone secretion rate was much lower in the three dogs classified as reactors than in the remaining four animals. The raised initial secretion and failure to react might have been related to an impaired general condition of these animals. In dog 55 some abnormality was suggested by the occurrence, before the dog was bled, of regular blood pressure waves which were slower than the respiratory rhythm (Mayer waves); whereas such waves are the normal accompaniment of haemorrhage, their occurrence in the early phase of the experiment is indicative of cerebral anoxia or some other pathological condition. In keeping with this interpretation, dog 55 did not tolerate haemorrhage; after the blood loss glucocorticoid secretion fell by 30 %, a sign of impaired

adrenocortical function. Dogs 99 and 102 had been bled 5·5 ml./kg before adrenal vein cannulation in order to obtain 'donor-blood' from the same animal. This was done, in the obviously wrong assumption, that it was too little to interfere with the basal conditions of the animal. In dog 107 Cheyne Stokes respiration developed, which may have been due to cerebral damage.

Table 2 contains information on eight dogs in which an attempt was made at lowering the initial secretion rate of aldosterone by giving the dogs a salt supplement and by reducing the operative stress; this was done by approaching the adrenal from the flank. The aldosterone values are corrected for losses.

TABLE 2. EFFECT OF HAEMORRHAGE ON ALDOSTERONE SECRETION

First adrenal blood sample (S_1) collected 30 min after end of dissection, second sample (S_2) after haemorrhage. Flank incision. (Steroids extracted from whole blood, aldosterone figures corrected for losses.)

dog no.	body wt. (kg)	adrenal blood sample no.	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change) aldosterone	cortisol	corticosterone	adrenal blood flow (ml./h)	mean b.p. (mmHg)	Mayer waves	Ht (% red cells)	blood withdrawn between S_1 and S_2 (ml./kg)	100 m-equiv. Na^+/day for (no. of days)
Group I. Reactors											
237, male	15·4	S_1	15·1	900	582	128	135	++	50	17	66
		S_2	21·7 + 44	782 − 13	455 − 22	124	95	++	53		.
240, female	21·0	S_1	7·7	867	109	164	150	−	66	20	14
		S_2	18·1 + 135	971 + 12	235 + 116	168	126	−	62		.
241, male	12·6	S_1	14·5	.	.	161	172	−	51	19	14
		S_2	27·1 + 87	1138 ·	836 ·	101	92	+	54		.
243, male	12·5	S_1	14·1	717	463	172	164	−	.	20	17
		S_2	23·6 + 67	641 − 11	381 − 18	136	132	+	53		.
mean ± S.E.		S_1	12·9 ± 1·7	828 ± 56	385 ± 142	156 ± 9·7	155 ± 8	·	·	19 ± 0·7	.
		S_2	22·6 ± 1·9	883 ± 109	477 ± 128	132 ± 14·0	111 ± 10				
Group II. Non-reactors											
242, male	26·5	S_1	12·0	572	619	548	190	−	79	20	20
		S_2	11·5 − 4	605 + 6	504 − 19	528	144	−	71		.
244, male	12·5	S_1	8·3	839	375	355	160	−	61	20	21
		S_2	8·6 + 4	811 − 3	326 − 13	264	104	−	55		.
245, male	12·0	S_1	11·9	768	513	245	152	−	47	20	16
		S_2	14·0 + 18	702 − 9	561 + 9	178	112	+	49		.
246, female	17·0	S_1	14·0	757	810	594	182	−	50	20	8
		S_2	11·0 − 21	594 − 22	405 − 50	399	101	+	52		.
mean ± S.E.		S_1	11·6 ± 1·2	734 ± 57	579 ± 92	436 ± 82	171 ± 9	·	·	20 ± 0	.
		S_2	11·3 ± 1·1	678 ± 51	449 ± 52	342 ± 77	115 ± 9·8				
				cortisol + corticosterone							
combined means groups I and II		S_1	12·2 ± 1·0	1271 ± 75		296 ± 65	163 ± 6·0	·	·	·	·
		S_2	17·0 ± 2·4	1256 ± 122		237 ± 54	113 ± 6·6				

In this series the initial aldosterone secretion was low in all dogs, 12·2μg (g adrenal)$^{-1}$ h^{-1} (corrected) being about equivalent to the amount of 3·9 (uncorrected) found in the reactors of table 1. Yet there was no response in four animals; an explanation can only be offered for dogs 242 and 246; dog 242 had a very high haematocrit and a blood pressure as high as 144 mmHg after haemorrhage. In this animal the withdrawal of blood might have constituted a therapeutic instead of a damaging measure. In dog 246 the fall of 36 % in glucocorticoid secretion after haemorrhage showed that there had been damage to the adrenal.

Aldosterone secretion following haemorrhage; 180 *min 'rest' after surgery*

The next experiments were designed to try whether a longer interval between the surgery and the taking of the first blood sample would improve the general condition of the dogs and thus increase the number responding to haemorrhage. In 10 dogs (table 3) 150 min rest were allowed between the end of the dissection and the injection of heparin which

TABLE 3. EFFECT OF HAEMORRHAGE ON ALDOSTERONE SECRETION

First adrenal blood sample (S_1) collected 180 min after end of dissection, second sample (S_2) after haemorrhage. Flank incision. (Steroids extracted from whole blood, aldosterone figures corrected for losses.)

dog no.	body wt. (kg)	adrenal blood sample no.	drenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change) aldosterone	cortisol	corticosterone	adrenal blood flow (ml./h)	mean b.p. (mmHg)	Mayer waves	blood withdrawn between S_1 and S_2 (ml./kg)	100 m-equiv. Na^+/day for (no. of days)
Group I. Reactors										
330, female	11·0	S_1	7·1	877	331	206	135	−	21	10
		S_2	15·5 +118	1108 +26	412 +24	150	130	−		.
335, male	11·8	S_1	8·9	877	351	166	155	−	28	3
		S_2	12·3 + 38	1038 +18	414 +18	130	115	+		.
337, female	12·7	S_1	19·5	1310	358	317	143	−	24	15
		S_2	27·1 + 39	1392 + 6	368 + 3	187	90	+		.
338, male	10·6	S_1	8·3	1094	302	237	125	−	25	16
		S_2	13·6 + 64	1484 +36	406 +34	188	100	+		.
339, male	12·9	S_1	5·9	1156	767	195	140	−	20	17
		S_2	9·0 + 53	1389 +20	661 −14	105	110	+		.
mean ± S.E.		S_1	9·9 ± 2·4	1063 ± 84	422 ± 87	224 ± 26	140 ± 5	.	25 ± 0·14	.
		S_2	15·5 ± 3·1	1282 ± 88	452 ± 53	152 ± 16	109 ± 7			
Group II. Non-reactors										
327, male	13·1	S_1	15·2	1722	954	240	175	−	21	42
		S_2	16·3 + 7	1638 − 5	720 −25	158	100	−		.
328, male	12·4	S_1	5·8	1046	571	424	170	−	21	32
		S_2	5·2 −10	1284 +23	659 +15	260	80	−		.
329, male	13·2	S_1	10·5	1258	1069	313	175	+	15	31
		S_2	4·2 −60	1160 − 8	545 −49	256	115	−		.
332, male	7·8	S_1	16·4	1304	535	150	140	−	23	9
		S_2	18·5 +13	1571 +20	545 + 2	113	95	+		.
333, male	14·0	S_1	13·3	1189	578	226	135	−	20	24
		S_2	14·1 + 6	1351 +14	611 + 6	267	115	+		.
mean ± S.E.		S_1	12·2 ± 1·9	1304 ± 113	741 ± 112	271 ± 46	159 ± 8·9	.	20 ± 1·3	.
		S_2	11·7 ± 2·9	1401 ± 89	616 ± 34	211 ± 32	101 ± 6·6	.		.
				cortisol + corticosterone						
combined means groups I and II		S_1	11·1 ± 1·5	1766 ± 147		247 ± 26	149 ± 5·8	.	.	.
		S_2	13·6 ± 2·1	1876 ± 87		181 ± 19	105 ± 4·7			

permitted the establishment of the shunt flow from the adrenal to the femoral vein. Another 30 min was allowed before collecting adrenal blood. As this group of experiments was also intended to serve as a control for experiments on nephrectomized dogs (see part II, p. 294), a minor intestinal operation was performed in order to simulate the surgical stress and manipulations of unilateral nephrectomy. A short piece of small intestine (dogs 327, 329, 330), a piece from the rectum (dog 328) or the tip of the caecum (dogs 332, 333, 335, 337, 338, 339) were removed.

The longer resting period did not consistently decrease the basal secretion rate of aldosterone. A rise in aldosterone secretion after haemorrhage was again present in only 50 % of the dogs. These dogs also showed rises in glucocorticoid secretion. With one exception in each group, the reactors had low and the non-reactors high initial aldosterone secretion rates. There were some anaemic dogs and some dogs with a high blood sedimentation rate in both groups; one of the non-reactors showed signs of circulatory collapse when bled only 15 ml./kg, whereas three of the reactors withstood the withdrawal of 24 to 28 ml./kg.

The cannulation of the adrenal vein is a severe stress and must lead to the release of *ACTH*. This *ACTH* release was not reduced, but enhanced, by the longer 'resting' period, as indicated by the higher secretion rate of glucocorticoids. From previous observations (Holzbauer 1964) it can be calculated that the *ACTH* secretion rate was more than doubled in the experiments with the longer rest. In spite of this, aldosterone secretion was not increased. This would suggest that the influence of some factor other than *ACTH* capable of stimulating aldosterone production was reduced during the longer resting period.

Sodium and potassium in plasma

Observations on the plasma concentrations of Na^+ and K^+ are summarized in table 4. There was no significant difference between the reactors and non-reactors. A slight rise in the plasma K^+ concentration (mean of 0·3 m-equiv./l.) was observed in all animals towards the end of the experiment. The mean plasma K^+ concentrations after a resting period of 180 min was 0·8 m-equiv./l. higher ($P < 0{\cdot}01$) than that found after a 30 min resting period.

TABLE 4. PLASMA CONCENTRATIONS OF Na^+ AND K^+ BEFORE AND AFTER HAEMORRHAGE IN DOGS UNDER CHLORALOSE ANAESTHESIA, SUBJECTED TO ADRENAL VEIN CANNULATION AND BLOOD COLLECTION

Plasma samples from femoral artery blood. A_1: immediately before first period of adrenal blood collection; A_2: after haemorrhage, and before collecting the second sample of adrenal blood; A_3: at the end of that collection. Means ± S.E.

group of dogs		arterial plasma sample no.	plasma concentration (m-equiv./l.) Na^+	K^+
dogs 237–246, table 2 (30 min after end of dissection)	'reactors'	A_1	142 ± 0·9	3·1 ± 0·20
		A_2	137 ± 0·6	3·4 ± 0·07
	'non-reactors'	A_1	143 ± 1·4	3·3 ± 0·23
		A_2	144 ± 1·7	3·5 ± 0·19
dogs 327–339, table 3 (180 min after end of dissection)	'reactors'	A_1	146 ± 1·3	4·0 ± 0·15
		A_2	144 ± 2·5	4·1 ± 0·15
		A_3	146 ± 1·2	4·3 ± 0·10
	'non-reactors'	A_1	152 ± 2·8	3·9 ± 0·10
		A_2	149 ± 1·6	4·2 ± 0·10
		A_3	156 ± 1·4	4·1 ± 0·20

Conclusions

Under our experimental conditions only 50 % of the dogs responded to haemorrhage with a rise in aldosterone secretion. Since these experiments were carried out, knowledge has accumulated on the stimulating effects of *ACTH* and angiotensin on the secretion of this hormone. The most likely explanation for the large number of non-reactors is a stimulation

of the release of these agents during the surgical procedures. This may sometimes be large enough to cause maximal aldosterone secretion and so to obscure the effect of haemorrhage. The fact that many non-reactors showed high basal secretion rates confirms this view. In some animals the initial aldosterone secretion may have been high as a result of a sodium-deficient diet, as will be shown in the next section. In the few instances in which failure to respond to haemorrhage occurred in spite of a low initial aldosterone secretion, the secretory capacity of the gland or the sensitivity to stimuli may have been subnormal. In animals with an impaired state of the circulation, there might have been damage to extra-adrenal structures which form a link in the mechanism under investigation, or interference with steroid synthesis caused by lack of oxygen or essential precursors. However, interference with steroid synthesis would be expected to affect glucocorticoids and aldosterone alike, so that this factor is excluded in all experiments in which glucocorticoid secretion showed no abrupt fall after haemorrhage.

II. Basal aldosterone secretion: effect of dietary sodium intake and of various surgical procedures

Table 5 illustrates the basal rates of aldosterone secretion obtained after surgical procedures of varying severity in dogs on low and high dietary sodium. The dogs are divided into twenty groups according to sodium intake and surgery performed. The daily potassium consumption was approximately 65 m-equiv. in all groups. The dietary changes did not affect plasma sodium concentrations, as estimated in femoral arterial blood before collection of the first sample of adrenal blood. In fact, the plasma sodium was lowest in the sodium-loaded dogs, but remained within the normal range. Laragh (1960) observed that in man, too, dietary sodium lack sufficient to cause an increase in aldosterone secretion was not associated with a low plasma sodium.

Table 5 confirms the dependence of aldosterone secretion on sodium intake. Among the dogs in which the only surgical procedure was adrenal vein cannulation through an abdominal midline incision (table 5, groups I to VI), the highest secretion rates of aldosterone were found in group I, the dogs which were kept for the longest time on the lowest sodium intake, and the lowest secretion rates in group VI, the dogs fed longest on the high sodium diet. The differences in the mean secretion rates are significant between groups I and II, I and IV and IV and VI (P between 0·05 and 0·01).

The type of operative procedures before adrenal blood collection also influenced aldosterone secretion rates. The mean aldosterone secretion rate of dogs in which the adrenal vein was approached from the midline (group IV) was significantly higher than that of dogs on the same sodium intake in which the incision was in the flank (groups VII and VIII), a procedure which greatly reduces traction on the viscera. After evisceration (groups XIV to XVI) and splanchnotomy (groups XVII to XX) there was also a general tendency for a raised initial aldosterone production. However, some of the groups are too small to allow any conclusions to be drawn.

Sodium deficient dogs in which both kidneys had been removed (group IX) did not show the elevated secretion rates of aldosterone found in dogs with intact kidneys (group I).

Variations in the sodium intake and different surgical procedures did not significantly affect the secretion rates of glucocorticoids.

TABLE 5. INFLUENCE OF SODIUM INTAKE ON BASAL SECRETION RATES OF CORTICOSTEROIDS (μg (g ADRENAL)$^{-1}$ h^{-1}, MEAN $\pm$ S.E.) IN DOGS SUBJECTED TO ADRENAL VEIN CANNULATION ALONE OR TO CANNULATION PRECEDED BY NEPHRECTOMY, EVISCERATION OR SPLANCHNOTOMY

(Steroids extracted from whole blood, aldosterone figures not corrected for losses.) n = no. of dogs. $F+B$ = cortisol + corticosterone.

dietary Na^+ intake/day (m-equiv. Na^+)	no. of days		operative procedure: midline incision (no additional operation)	right flank incision (no additional operation)	left flank incision (no additional operation)	midline incision: bilateral nephrectomy	midline incision: right nephrectomy	midline incision: evisceration	midline incision: bilateral splanchnotomy
30*	6–84 (mean: 26)	group no.	I	.	.	IX	.	XIV	XVII
		n	13			4		1	1
		aldosterone	10·8 ± 1·0			6·3 ± 2·2		10·9	10·8
		$F+B$	1627 ± 82			1754 ± 134		1816	1422
30	1–5 (mean: 2)	group no.	II	.	.	X	XIII	.	XVIII
		n	4			5	4		7
		aldosterone	6·9 ± 0·7			7·1 ± 1·3	8·8 ± 1·1		9·9 ± 2
		$F+B$	1361 ± 65			1487 ± 75	1932 ± 133		1532 ± 72
100	1–5 (mean: 4)	group no.	III	.	.	XI	.	.	.
		n	3			1			
		aldosterone	7·1 ± 1·8			6·4			
		$F+B$	1705 ± 152			1565			
100	7–31 (mean: 20)	group no.	IV	VII	VIII	XII	.	XV	XIX
		n	15	6	8	5		12	3
		aldosterone	7·4 ± 1·0	4·8 ± 0·8	3·8 ± 0·5	7·1 ± 1·2		9·8 ± 1·0	12·4 ± 0·4
		$F+B$	1637 ± 115	1518 ± 97	1467 ± 109	1579 ± 176		1640 ± 136	2084 ± 180
170 followed by 100	5–22 (mean: 12) 24–38 (mean: 29)	group no.	V	.	.	.	.	XVI	.
		n	5					2	
		aldosterone	5·8 ± 0·7					8·6, 12·7	
		$F+B$	1616 ± 112					1709, 1208	
170	4–9 (mean: 7)	group no.	VI	.	.	.	.	.	XX
		n	4						2
		aldosterone	3·9 ± 1·0						4·1, 4·4
		$F+B$	1370 ± 128						1457, 1091

* 60 m-equiv. is considered to be a normal daily sodium intake for an average size dog.

III. THE EFFECT OF EVISCERATION ON THE RESPONSE OF ALDOSTERONE SECRETION TO HAEMORRHAGE

These experiments were carried out in order to see whether the removal of viscera interferes with the rise in aldosterone secretion after haemorrhage.

Procedures and observations

The intestinal tract, the spleen and the pancreas were removed and the blood supply to the liver (from portal vein and hepatic arteries) interrupted before the adrenal vein was cannulated. Collection of a control sample of adrenal venous blood was started 40 to 120 min after evisceration (20 to 100 min after injection of heparin and adrenal vein cannulation); then the dogs were bled and a second adrenal blood sample was taken.

Preliminary experiments were carried out in three dogs at a time when the steroids were still being extracted from plasma. The amount of aldosterone and of glucocorticoids found in the adrenal venous plasma was very low. Aldosterone secretion rates were 3·5, $<0·2$ and 2·2 μg (g adrenal)$^{-1}$ h^{-1} before haemorrhage and rose after blood loss to 4·9, 1·3 and 5·1 μg respectively.

The results obtained by analysing whole blood samples from 14 eviscerated dogs are summarized in table 6. Only two of 14 dogs responded to haemorrhage with a rise in aldosterone secretion of more than 30 %. As discussed earlier, eviscerated animals showed a tendency to higher initial secretion rates of aldosterone than controls on the same sodium intake, irrespective of the time of rest allowed after completion of surgery. Accordingly, among the 12 non-reactors, 10 had initial secretion rates higher than the mean of the non-eviscerated groups on the same diet, in one (192) the rate was equal, in one (203) it was less. In the two reactors the rate was equal to that of the non-eviscerated controls.

TABLE 6. THE EFFECT OF HAEMORRHAGE ON ALDOSTERONE SECRETION IN EVISCERATED DOGS

Collection of first adrenal blood sample (S_1) started between 40 and 120 min after evisceration, second sample (S_2) after haemorrhage. (Steroids extracted from whole blood, aldosterone figures not corrected for losses.)

dog no.	body weight (kg)	blood sample no.	time after evisceration (min)	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change) aldosterone	cortisol + corticosterone	adrenal blood flow (ml./h)	mean b.p. (mmHg)	Mayer waves	blood withdrawn between S_1 and S_2 (ml./kg)	Na^+ intake (m-equiv./day)
Group I. Reactors										
182, male	7·6	S_1	75	7·6	1955	276	142	·	12	100 for more than 7 days (as group IV, table 5)
		S_2		14·8 +95	2293 +17	139	78	·		
196, male	8·5	S_1	75	7·2	1156	254	180	–	19	
		S_2		9·7 +35	854 −26	247	105	–		
Group II. Non-reactors										
159, male	9·1	S_1	78	10·9	1816	144	114	+	10	30 m-equiv. for 28 days (as group I, table 5)
		S_2		11·4 + 5	1438 −21	76	60	++		
176, male	13·1	S_1	72	8·6	1709	338	123	–	12	170 for 18 days followed by 100 for 31 and 38 days (as group V, table 5)
		S_2		8·4 − 2	1533 −10	199	62	–		
179, male	16·8	S_1	84	12·7	1208	190	92	–	14	
		S_2		12·3 − 3	1697 +40	180	64	+		
160, female	7·6	S_1	44	10·7	1856	341	171	–	18	100 for more than 7 days (as group IV, table 5)
		S_2		8·3 −22	1395 −25	151	74	–		
161, female	8·1	S_1	41	14·1	2533	336	140	–	21	
		S_2		12·4 −12	2489 − 2	154	78	–		
184, female	7·0	S_1	93	11·9	1450	228	164	–	8	
		S_2		12·9 + 8	1670 +15	106	90	–		
187, female	10·4	S_1	67	8·4	1328	302	165	–	19	
		S_2		8·5 + 1	1221 − 8	295	113	–		
192, male	15·6	S_1	72	7·2	1130	197	176	+	14	
		S_2		7·2 0	1063 − 6	211	94	+		
203, female	12·5	S_1	120	3·4	1268	192	144	–	16	
		S_2		3·5 + 3	1150 − 9	116	80	+		
206, female	11·3	S_1	120	10·0	1620	411	146	–	20	
		S_2		10·0 0	1577 − 3	174	88	–		
209, female	7·3	S_1	117	9·7	1660	245	170	–	14	
		S_2		12·2 +26	1591 − 4	178	85	–		
210, male	12·4	S_1	113	15·4	2418	160	159	+	13	
		S_2		19·9 +29	1958 −19	82	70	+		

Table 7 summarizes some circulatory data and the plasma electrolyte changes in the eviscerated dogs. The mean blood loss tolerated was only 15 ml./kg as compared with 20 or more when no evisceration had been performed. The plasma potassium concentration tended to be higher than in the non-eviscerated dogs with similar resting periods (compare table 4).

Conclusions

In eviscerated dogs, the failure of aldosterone secretion to respond with a rise to the stimulus of haemorrhage appears to be linked with high basal secretion rates of the hormone. The most likely explanation is an increase of the release of aldosterone-stimulating factors by the stress of the evisceration. Interference with steroid anabolism by excluding the liver (Ayers, Davis, Lieberman, Carpenter & Berman 1962) is another possibility. However, this should not prevent further rises, in view of the fact that there is no evidence for an aldosterone feedback mechanism within the adrenal gland (Blair-West, Coghlan & Denton 1962). Hypothetical receptors in the abdominal vascular bed forming part of a reflex pathway which inhibits aldosterone secretion and which evisceration would remove would not be incompatible with the results.

TABLE 7. MEAN VALUES AND STANDARD ERRORS OF ARTERIAL BLOOD PRESSURES, HAEMATOCRITS AND PLASMA CONCENTRATIONS OF SODIUM AND POTASSIUM BEFORE AND AFTER HAEMORRHAGE IN EVISCERATED DOGS

adrenal venous blood sample	mean b.p. (mmHg)	adrenal blood flow (ml./h)	arterial blood sample taken	haematocrit (% red cells)	plasma concentration (m-equiv./l.) Na^+	plasma concentration (m-equiv./l.) K^+
S_1 control	$149 \pm 6{\cdot}8$	258 ± 21	before S_1	$58 \pm 1{\cdot}9$	145 ± 1	$3{\cdot}9 \pm 0{\cdot}1$
S_2 after haemorrhage	$82 \pm 4{\cdot}2$	165 ± 16	at end of haemorrhage	$54 \pm 1{\cdot}4$	145 ± 1	$4{\cdot}5 \pm 0{\cdot}2$
.	.	.	at end of experiment	$53 \pm 1{\cdot}3$	147 ± 2	$4{\cdot}6 \pm 0{\cdot}2$

IV. PROPRIOCEPTORS IN THE CAROTID VASCULAR BED AND ALDOSTERONE SECRETION

The suggestion that the carotid sinus may contain receptors for a reflex arc which is involved in the control of aldosterone secretion was first made by Barger, Muldowney & Liebowitz (1959). In the same year, Bartter, Mills, Biglieri & Delea (1959) reported that in nine dogs, which were anaesthetized and under the stress of adrenal vein cannulation, compression of the carotid artery low in the neck caused a significant rise in aldosterone secretion. This effect was no longer present when the carotid and thyroid arteries had been denervated 3 to 11 days previously. Biglieri & Ganong (1961) attributed the rise to a release of *ACTH* since it disappeared after hypophysectomy.

Bartter, Mills & Gann (1960) reported that the rise in aldosterone secretion which follows constriction of the thoracic vena cava is prevented by denervation of the carotid sinuses along with denervation of the origin of the thyroid arteries, a region which they found to contain additional pressure sensitive structures in the dog (Gann & Bartter 1959). In fact, in their hands, denervation of this region alone was sufficient to suppress the response. In a detailed study Carpenter, Davis & Ayers (1961 *a*) were unable to confirm these findings. They came to the conclusion that the carotid or aortic baroreceptors are not involved in the mechanism which leads to a rise in aldosterone secretion after constriction of the thoracic inferior vena cava. In this section the response of aldosterone secretion to acute haemorrhage is studied after denervation of carotid sinuses and thyroid arteries.

Procedures and observations

In a preliminary aseptic operation both sinus nerves were cut, the region of both carotid sinuses stripped of nerve plexuses and adventitial tissue, and the two thyro-carotid junctions carefully stripped in a similar way. The adrenal blood collection was carried out 14 to 27 days later. All dogs were hypertensive. A control sample of adrenal venous blood was collected for 20, 25 or 30 min half an hour after the dissection had been completed, then 14 to 27 ml./kg of blood were withdrawn from a femoral artery and a second sample of blood was taken 5 min later. When artificial respiration was used, the stroke of the pump was increased by about 10 % during this period. At the end of each experiment the blood withdrawn during the haemorrhage was re-infused and the effect of carotid occlusion on the blood pressure was checked before and after vagotomy.

The results obtained are shown in tables 8A and B. Experiments were done on 14 dogs. In nine of these (table 8A) the Hering reflex was completely absent at the end of the experiments. In the remaining dogs vestiges of this reflex were observed after bilateral vagotomy, but the high initial blood pressures indicated that denervation must have been quite extensive.

Out of nine dogs, in which denervation was complete, five dogs responded to haemorrhage with rises in aldosterone secretion varying from 40 to 124 % whereas their glucocorticoid secretion remained unchanged (−12 to +16 %). In the remaining four dogs aldosterone secretion did not change but glucocorticoid secretion was slightly decreased, and once (dog 297) sufficiently to suggest failure of steroid synthesis. Out of five dogs in which denervation might have been incomplete, dog 295 responded to haemorrhage with a rise of aldosterone secretion by 73 %; its glucocorticoid secretion was unchanged (−7 %). The other four did not show significant changes in aldosterone secretion but there was some rise in glucocorticoid secretion. Reactors and non-reactors did not differ by the height of their blood pressure either before or after haemorrhage, they tolerated withdrawal of blood equally well (see means of table 8B), and they showed no difference in haematocrit or plasma electrolyte concentration. There was, however, a clear difference between the mean initial secretion rate of aldosterone, which was 11·7 in the reactors and 18·4 in the non-reactors. Owing, however, to one exception in each group, the difference between the means is not statistically significant. The exception among the reactors, dog 305, had the unusually high initial secretion rate of aldosterone of 27·2 μg (g adrenal)$^{-1}$ h^{-1}, and yet secretion increased further after bleeding. Dog 305 was also the only one which had Mayer waves before haemorrhage. The exception among the non-reactors, dog 283, stands out, together with 305, by an exceptionally high initial secretion of glucocorticoids; this may indicate prolonged pre-operative stimulation of the pituitary and suggest some chronic abnormal state. It is interesting that only one of the two dogs (305) showed both the glucocorticoids and aldosterone raised in parallel. Table 8B also indicates that complete sinus denervation (groups I and II) is accompanied by a tendency to high glucocorticoid secretion, a fact which is not unexpected since the dogs must be in a state of chronic stress.

Conclusions

Denervation of the baroreceptors and chemoreceptors in the carotid region did not abolish the rise in aldosterone secretion following haemorrhage, the proportion of reacting

TABLE 8. EFFECT OF HAEMORRHAGE ON ALDOSTERONE SECRETION 14 TO 27 DAYS AFTER BILATERAL DENERVATION OF THE CAROTID SINUSES

First adrenal blood sample (S_1) collected 30 min after end of dissection, second sample (S_2) after haemorrhage. Flank incision. (Steroids extracted from whole blood, aldosterone figures corrected for losses.)

dog no.	body weight (kg)	sample no.	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change): aldosterone	cortisol + corticosterone	mean b.p. (mmHg)	Mayer waves	blood withdrawn between S_1 and S_2 (ml./kg)
A. DOGS IN WHICH DENERVATION WAS FOUND TO BE COMPLETE							
Group I. Reactors							
279, male	15·4	S_1	10·6	1591	220	−	21
		S_2	23·7 +124	1853 +16	135	−	
282, female	9·1	S_1	11·0	1703	135	−	23
		S_2	16·5 + 50	1506 −12	88	+	
294, male	13·1	S_1	5·5	820*	187	−	14
		S_2	11·8 +115	869* +6	107	+	
298, female	18·0	S_1	7·5	1024	165	−	21
		S_2	13·3 + 77	1186 +16	80	+	
305, female	10·6	S_1	27·2	2167	170	+	27
		S_2	38·2 + 40	2175 0	95	+	
Group II. Non-reactors							
280, male	15·8	S_1	18·8	1287	162	−	19
		S_2	17·5 −7	1109 −14	80	+	
281, male	12·4	S_1	31·0	1945	180	−	24
		S_2	32·2 +4	1398 −28	143	−	
283, male	11·3	S_1	9·0	2564	203	−	20
		S_2	9·2 +2	2000 −22	112	−	
297, male	21·0	S_1	12·9	1940	190	−	21
		S_2	12·1 −6	1039 −46	80	+	
B. DOGS IN WHICH DENERVATION WAS POSSIBLY INCOMPLETE							
Group III. Reactor							
295, female	13·4	S_1	8·2	1525	176	−	20
		S_2	14·2 +73	1420 −7	104	+	
Group IV. Non-reactors							
292, female	14·3	S_1	13·4	1290	197	−	20
		S_2	16·1 +20	1420 +10	90	+	
293, male	17·8	S_1	16·2	818*	178	−	20
		S_2	16·9 + 4	1386* +69	100	+	
296, female	13·8	S_1	26·0	1865	182	−	18
		S_2	26·3 + 1	2352 +26	80	+	
306, male	14·3	S_1	19·9	1060	165	−	26
		S_2	24·2 +22	1279 +21	135	+	
means of groups I+III (n = 6)		S_1	11·7 ± 3·2	1602 ± 183	176 ± 11	·	21 ± 1·7
		S_2	19·6 ± 4·1	1628 ± 174	102 ± 8		
means of groups II+IV (n = 8)		S_1	18·4 ± 2·6	1707 ± 198	182 ± 5	·	21 ± 0·9
		S_2	19·3 ± 2·7	1514 ± 183	103 ± 9		
means of all groups (n = 14)		S_1	15·5 ± 2·1	1663 ± 133	179 ± 6	·	21 ± 0·9
		S_2	19·4 ± 2·2	1561 ± 125	102 ± 6		

* Cortisol only.

dogs (6 out of 14) being about normal. Therefore, in animals with intact pituitary glands and kidneys, the part played in this phenomenon by a postulated aldosterone regulating system with receptor sites in the carotid arteries, cannot be a crucial one.

V. Effect of splanchnotomy on the response to haemorrhage

These experiments were designed to test whether haemodynamic or other changes taking place in the splanchnic bed during haemorrhage might elicit a reflex which could accelerate aldosterone secretion and which would use the splanchnic nerves as its pathway.

Since the work by Gammon & Bronk (1935) it is known that there are receptors in the splanchnic area which respond to changes in blood flow. Reflexes elicited by their activity seem to be of little importance in the regulation of the systemic blood pressure (Heymans, de Schaepdryver & de Vleeschhouwer 1960) but may be concerned with local distribution of blood in abdominal viscera (Heymans & Neil 1958). The experiments tried to establish whether unilateral or bilateral splanchnotomy alter either basal aldosterone secretion or its rise elicited by haemorrhage. Adrenocortical secretion rates were also measured before, during and after electrical stimulation of the central ends of the sectioned splanchnic nerves.

Procedures and observations

In the four groups of experiments the abdomen was opened by a midline incision, the greater and lesser splanchnic nerves were dissected out at their emergence from the diaphragm and ligatures placed under the nerves. Then the left adreno-lumbar vein was dissected for cannulation. Adrenal blood samples were usually collected for 30 min.

Unilateral splanchnotomy and haemorrhage

In four dogs, one of which was eviscerated, the left splanchnic nerves were sectioned before the collection of a control sample from the left adrenal. Then the dogs were bled and a second sample of adrenal venous blood was taken. Steroids were extracted from plasma in one, from whole blood in three experiments.

With the exception of one dog, in which secretion was already high before haemorrhage, bleeding raised the rate of aldosterone secretion. Glucocorticoids were not affected.

Acute bilateral splanchnotomy and haemorrhage

In a preliminary group of five dogs (table 9), four of which were eviscerated, a control sample of adrenal venous blood was taken 41 to 70 min after bilateral splanchnotomy. Then the blood volume was expanded by an infusion of donor blood (20 to 23 ml./kg over 10 min) and a second blood sample collected. This was followed by withdrawal of blood (5 to 23 ml./kg over 10 min) and collection of a last blood sample. In the non-eviscerated dog the initial expansion of the blood volume was omitted. Steroids were extracted from plasma alone, no correction for losses were made.

In three dogs expansion of the blood volume caused a fall in aldosterone secretion of 25, 32 and 15 %, whereas in the fourth dog which had an initial secretion rate of only 1·7 μg (g adrenal)$^{-1}$ h^{-1} the value rose to 2·9 μg. In no instance was haemorrhage followed by a significant rise in aldosterone secretion. Glucocorticoid secretion rates tended to fall during

the course of the experiments. Adrenal blood flow after haemorrhage was nearly halved. The initial blood pressure was lower than usual and the amount of blood withdrawn was less than control dogs tolerated.

The second series of experiments was carried out on five dogs in which the dietary salt intake was controlled. None of these dogs was eviscerated. A control sample of adrenal blood was collected 55 to 112 min after sectioning the splanchnic nerves on both sides. Then the dogs were bled (8 to 18 ml./kg) and a second sample was taken. Steroids were estimated in extracts from whole blood. The aldosterone figures were not corrected for losses.

TABLE 9. EFFECT OF CHANGES IN BLOOD VOLUME ON ALDOSTERONE SECRETION IN DOGS AFTER ACUTE BILATERAL SPLANCHNOTOMY

(Steroids extracted from plasma only, aldosterone figures not corrected for losses.)

dog no.	adrenal blood sample	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1}) aldosterone	cortisol + corticosterone	adrenal blood flow (ml./h)	mean b.p. (mmHg)	blood infused (ml./kg)	blood withdrawn (ml./kg)
46–49, mean ± S.E. (1 female, 3 males) eviscerated	44–70 min after bilateral splanchnotomy	6·0 ± 1·8	600 ± 62	305 ± 35	116 ± 15	21 ± 0·7	
	immediately after infusion of donor blood	4·8 ± 0·9	529 ± 104	359 ± 77	122 ± 13		14 ± 1·1
	5 min after haemorrhage	4·7 ± 0·8	434 ± 85	191 ± 34	70 ± 14		
54, male, not eviscerated	41 min after bilateral splanchnotomy	1·5	550	136	110		9
	5 min after haemorrhage	1·7	513	71	65		

The results are seen in table 10. The group includes animals with low and high Na^+ intake. None of the dogs responded to haemorrhage with a significant rise in aldosterone secretion. With the exception of dog 143 the initial secretion rates of aldosterone were higher than the mean of the corresponding control groups on the same diet (see table 5). The dogs showed some fall in glucocorticoid secretion. Initial blood pressures were low. The amount of blood which could be withdrawn during haemorrhage without causing a circulatory collapse averaged only 12·6 ml./kg. During collection of the second adrenal blood sample the mean arterial pressure was below 60 mmHg in three of the five dogs. The adrenal blood flow was about halved. The mean plasma K^+ concentration was slightly elevated, and rose by a further 1·6 m-equiv./l. in the course of the experiment.

These experiments might be interpreted in two ways. Either severing of the splanchnic nerves interfered with a mechanism which leads to a rise in aldosterone secretion following haemorrhage, or the failure to observe a rise was due to the poor state of the acutely splanchnotomized animal: table 10 shows that the circulation was impaired, haemorrhage was badly tolerated, and initial secretion rate of aldosterone was high. The first alternative

would imply that under normal circulatory conditions the splanchnic nerves conduct impulses from abdominal proprioceptors which may either inhibit an 'aldosterone stimulating' or stimulate an 'aldosterone inhibiting' mechanism. If stretch receptors were responsible, bilateral splanchnotomy should raise, and stimulation of the central end of the nerve should lower aldosterone secretion. If chemoreceptors were involved, which started to fire after blood loss, section of the nerves should not affect secretion rate but stimulation of the central ends should raise it.

These different possibilities are tested in the next sections.

TABLE 10. EFFECT OF HAEMORRHAGE ON ALDOSTERONE SECRETION IN ACUTELY SPLANCHNOTOMIZED DOGS

Collection of first adrenal blood sample (S_1) started 55 to 112 min after splanchnotomy and 10 to 60 min after end of dissection; second sample (S_2) taken after haemorrhage. Arterial blood samples taken at start of S_1 (A_1), end of haemorrhage (A_2), and end of S_2 (A_3). (Steroids extracted from whole blood, aldosterone figures not corrected for losses.)

dog no.	body weight (kg)	adrenal blood sample no.	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change) aldosterone	cortisol + corticosterone	adrenal blood flow (ml./h)	mean b.p. (mmHg)	Mayer waves	blood withdrawn (ml./kg)	arterial blood sample no.	Ht (% red cells)	plasma conc. (m-equiv./l.) Na^+	K^+	Na^+ intake
164, female	16·7	S_1	12·7	2357	180	122	−	9	A_1	59	142	4·7	100 m-equiv. Na^+/day for more than 7 days
		S_2	12·4 − 2	1981 − 16	96	57	−		A_2	59	137	6·5	
									A_3	57	135	6·6	
165, female	19·1	S_1	13·0	2151	312	124	−	15	A_1	52	144	3·8	
		S_2	15·1 + 16	2091 − 3	174	82	−		A_2	52	132	4·7	
									A_3	48	144	5·0	
143, male	17·4	S_1	10·8	1422	240	153	−	13	A_1	65	.	.	30 m-equiv. Na^+/day for 26 days
		S_2	10·6 − 2	1372 − 4	86	70	−		A_2	58	.	.	
									A_3	.	.	.	
169, male	12·6	S_1	4·1	1457	158	100	+	8	A_1	54	138	4·1	170 m-equiv. Na^+/day for 5 days
		S_2	5·3 + 29	1159 − 20	64	52	+		A_2	52	137	4·6	
									A_3	48	139	5·0	
170, male	8·4	S_1	4·4	1091	234	79	−	18	A_1	50	139	4·1	
		S_2	4·8 + 9	1024 − 6	110	44	+		A_2	49	139	4·7	
									A_3	48	143	5·6	

Bilateral splanchnotomy and basal steroid secretion

In seven dogs, one of which was eviscerated, a control sample of adrenal venous blood was collected first, then the splanchnic nerves were severed and a few minutes later a second sample was taken. Steroids were extracted from whole blood, no correction was made for losses.

Table 11 shows that there was no difference in the rates of secretion of aldosterone or glucocorticoids before and after sectioning of the nerves. In five out of seven dogs the initial secretion rate of aldosterone was higher than the mean observed in control dogs which were studied at the same time. This might have obscured rises which could be expected if the splanchnic nerves were transmitting baroreceptor activity. An attempt at imitating the effect of afferent impulses was made in the experiments of the next section.

Electrical stimulation of splanchnic nerves

Six dogs were eviscerated and the splanchnic nerves sectioned bilaterally between ligatures. In two dogs, sectioning of the splanchnic nerves was carried out before evisceration,

in an attempt at obtaining lower initial aldosterone secretion by reducing the stressful afferent impulses elicited by ligation of the abdominal arteries. Though secretion rate was indeed low in these experiments, it was also low in three instances in which the order of the operative procedures had not been reversed.

The central stumps of the nerves were placed on shielded platinum electrodes and a first blood sample collected. Stimulation of the nerves was started 2 to 10 min before, and continued throughout the collection of the second blood sample. It was carried out for 30 s in every minute with rectangular pulses of 10 ms duration at a frequency of 33/s and a voltage ranging from 5 to 9 V.

TABLE 11. EFFECT OF ACUTE BILATERAL SPLANCHNOTOMY ON ADRENAL STEROID SECRETION

Means ± S.E. of observations made on dogs 60 to 67. Sample 1 (S_1) before, sample 2 (S_2) 2 to 15 min after splanchnotomy. (Steroids extracted from whole blood, aldosterone figures not corrected for losses.)

	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1})		mean b.p.		adrenal blood flow	
adrenal blood sample no.	aldosterone	cortisol + corticosterone	(mmHg)	% change	(ml./h)	% change
S_1	11·9 ± 1·9	1145 ± 94	128 ± 6	−17	221 ± 29	−46
S_2	10·9 ± 2·9	1075 ± 86	106 ± 6		119 ± 14	

Electrical stimulation of the central ends of the sectioned splanchnic nerves twice produced rises, twice falls and 4 times no change in aldosterone secretion. The rises, which were quite large, were associated with a reduction by 50 % in adrenal blood flow, due to a fall in mean blood pressure in the first, and to high-amplitude Mayer waves in the second dog. The other dogs showed but small changes in adrenal blood flow during the period of stimulation. It is obviously not justifiable to attribute the rises in aldosterone production in two dogs to the stimulation of the splanchnic nerves when impairment of the circulation would easily account for the increases.

In all experiments, glucocorticoid secretion was little affected. The blood pressure records became very irregular during stimulation. Sometimes sharp rises occurred during the 30 s when stimulation was on, sometimes during the intervals. Occasionally stimulation of the nerves on one side seemed to influence the systemic pressure more than stimulation of the contralateral nerves. Onset of stimulation often caused muscular jerks or respiratory arrest. In conclusion, the results of the experiments in which aldosterone secretion was measured before and after acutely performed splanchnotomy or during electrical stimulation of the central end of the severed nerves did not lend support to the theory that splanchnic activity exerted a specific control over aldosterone secretion.

Aldosterone secretion after haemorrhage in dogs subjected to chronic bilateral splanchnotomy

It was obvious that the failure to obtain a rise in aldosterone secretion by bleeding acutely splanchnotomized dogs would defy interpretation as long as the circulatory embarrassment resulting from the procedure complicated the picture. The splanchnic nerves were therefore sectioned in a preliminary aseptic operation and the response to haemorrhage was tested 9 to 20 days later when the dogs had completely recovered.

Bilateral splanchnotomy was performed in 10 dogs under pentobarbitone sodium anaesthesia. After an interval of at least 9 days the left adreno-lumbar vein was cannulated

via a flank incision. A control sample was collected 30 min later, the dog was bled (9 to 20 ml./kg), and a second sample was taken. Steroids were extracted from whole blood and the aldosterone figures were corrected for 100 % recovery.

TABLE 12. EFFECT OF HAEMORRHAGE ON ALDOSTERONE SECRETION IN DOGS SPLANCHNOTOMIZED BILATERALLY 9 TO 20 DAYS BEFORE THE EXPERIMENT

All dogs received 100 m-equiv. Na^+/day for more than 16 days. First adrenal blood sample (S_1) collected 30 min after end of dissection, second sample (S_2) after haemorrhage. Arterial blood samples taken from femoral artery: A_1, end of dissection; A_2, end of haemorrhage; A_3, end of experiment. Flank incision. (Steroids extracted from whole blood, aldosterone figures corrected for losses.)

dog no.	body wt. (kg)	adrenal blood sample no.	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change) aldosterone	cortisol + corticosterone	adrenal blood flow (ml./h)	mean b.p. (mmHg)	Mayer waves	blood withdrawn (ml./kg)	arterial blood sample no.	Ht (% red cells)	plasma conc. (m-equiv./l.) Na^+	K^+
Group I. Reactors												
267, male	9·8	S_1	10·6	.	240	120	−	18	A_1	37	143	3·6
		S_2	21·0 + 98	1496 ·	112	80	+		A_2	41	147	3·5
									A_3	38	146	3·7
268, male	13·0	S_1	14·4	1773	262	157	−	9	A_1	51	146	3·4
		S_2	35·2 + 144	2524 + 42	173	80	−		A_2	52	141	3·4
									A_3	48	146	4·3
272, female	9·1	S_1	8·7	1437	254	150	−	20	A_1	50	145	3·5
		S_2	12·4 + 43	1374 − 4	103	90	+		A_2	50	140	3·8
									A_3	50	143	3·1
273, female	7·2	S_1	10·8	.	384	140	−	20	A_1	57	143	3·3
		S_2	18·7 + 73	1092 ·	192	70	−		A_2	55	140	3·6
									A_3	51	143	3·8
274, male	13·0	S_1	9·5	1445	285	150	−	20	A_1	42	148	3·3
		S_2	12·6 + 33	1588 + 10	165	85	+		A_2	45	146	3·1
									A_3	41	143	3·5
276, female	8·9	S_1	10·5	1374	214	150	−	16	A_1	59	158	3·1
		S_2	22·1 + 110	1499 + 9	126	80	+		A_2	56	152	3·9
									A_3	53	152	3·7
277, male	11·6	S_1	7·1	1814	166	140	−	17	A_1	48	150	3·8
		S_2	10·8 + 52	1514 − 17	96	80	+		A_2	47	138	3·5
									A_3	48	137	3·3
mean ± S.E.		S_1	10·2 ± 0·9	1569 ± 93	258 ± 26	144 ± 4·6		17 ± 0·2	A_1	49 ± 2·9	148 ± 2·0	3·4 ± 0·1
		S_2	19·0 ± 3·2	1584 ± 168	138 ± 14	81 ± 2·3	·		A_2	49 ± 2·1	143 ± 1·9	3·5 ± 0·1
									A_3	47 ± 2·1	144 ± 1·7	3·6 ± 0·1
Group II. Non-reactors												
266, female	8·1	S_1	34·4	.	202	130	−	20	A_1	38	143	3·6
		S_2	21·4 − 38	1622 ·	101	85	+		A_2	45	141	2·6
									A_3	44	145	4·0
270, female	13·4	S_1	15·5	1629	295	140	−	20	A_1	45	148	3·4
		S_2	14·8 − 5	1666 + 2	180	100	−		A_2	50	148	3·5
									A_3	45	145	3·7
271, male	9·5	S_1	16·4	1160	112	130	−	16	A_1	53	143	3·2
		S_2	20·2 + 23	1306 + 13	46	80	−		A_2	43	139	4·6
									A_3	44	145	3·7
mean ± S.E.		S_1	22·1 ± 6·2	1629, 1160	203 ± 53	133 ± 3·3		19 ± 1·4	A_1	45 ± 4·3	145 ± 1·7	3·4 ± 0·1
		S_2	18·8 ± 2·0	1531 ± 113	109 ± 39	88 ± 6·0	·		A_2	46 ± 2·1	143 ± 2·7	3·6 ± 0·2
									A_3	44 ± 0·4	145 ± 0	3·8 ± 0·1
combined results group I + II mean ± S.E.		S_1	13·8 ± 2·5	1519 ± 88	241 ± 24	141 ± 3·6		18 ± 1·1	A_1	48 ± 2·4	147 ± 1·5	3·4 ± 0·07
		S_2	18·9 ± 2·2	1568 ± 119	129 ± 15	83 ± 2·4	·		A_2	48 ± 1·6	143 ± 1·5	3·6 ± 0·16
									A_3	46 ± 1·5	145 ± 1·1	3·7 ± 0·1

As can be seen from table 12, satisfactory circulatory conditions were maintained throughout the experiment in all but one dog. Out of the 10 splanchnotomized dogs seven responded to haemorrhage with an increase in aldosterone secretion of more than 30 %. The initial aldosterone secretion rates of the reactors were below 11 μg (g adrenal)$^{-1}$ h^{-1} with the exception of dog 268 in which it was 14·4 μg. In the non-reactors it was higher with a mean of 22·1. In both groups of dogs the secretion rates of the glucocorticoids changed very little after haemorrhage. The initial blood pressure was not lower than usual, and the lowest figure after haemorrhage was 70 mmHg (dog 273). The decrease in adrenal blood flow after bleeding was of the same order of magnitude as in dogs with intact splanchnic nerves. The concentration of K^+ in peripheral plasma at the end of the dissection was similar to that in normal dogs. There was a mean rise in K^+ of 0·3 m-equiv./l. in the course of the experiment. All reactors had an uneventful recovery period after the operation, although dog 267 was anaemic at the time of the experiment (Ht 37 %), and dog 272 had a high blood sedimentation rate. Among the non-reactors dog 266 was anaemic (Ht 38 %). Dog 270 had been suffering from diarrhoea for 2 days after the operation. Dog 271 bled profusely from a spleen injury during the dissection and lost, in spite of infusion of donor blood, 7·8 ml./kg before the intentional haemorrhage.

One dog (275) has not been included in table 12 but deserves mention because it showed an extremely low initial secretion rate of aldosterone (2·5 μg (g adrenal)$^{-1}$ h^{-1}). This was probably the result of its being kept on the high sodium intake for a period of over 3 months. Among other dogs kept for similar periods on the same diet some, but not all, showed extreme depression of aldosterone secretion (see section on anoxia). It appears that dogs which respond in this way have also lost the capacity of increasing aldosterone production to normal levels during acute demands. Dog 275 apparently accelerated its aldosterone secretion by 30 % after bleeding, but since this represented an absolute increase of only 0·15 μg (in 25 min), the readings could not be relied on and the results have been disregarded.

The difference between the acute and chronic effect of splanchnotomy on secretion of aldosterone is not related to adrenal blood flow. This is shown in figure 3 in which adrenal effluent (which, of course, may include some extra-adrenal blood) is plotted against the time elapsed after splanchnotomy. Flow was smallest during collection periods starting 2 to 15 min after section of the nerves, but was back to normal 60 to 120 min after splanchnotomy. The blood pressure was hardly changed during the recovery of flow.

Conclusions

No evidence was obtained for a specific link between splanchnic nerve activity and aldosterone secretion. The possibility of such a relation had been suggested by the failure of acutely splanchnotomized dogs to respond to haemorrhage with a rise in aldosterone secretion. However, this effect was found to be unspecific and connected with the deterioration in the condition of the circulation after acute splanchnotomy. The factor responsible was not the reduction of adrenal blood flow, since this was present for the first 10 to 40 min only after severing the nerves, and reverted to normal at about 1 h. During the period of reduced blood flow aldosterone secretion was high and glucocorticoid secretion normal. Intolerance to haemorrhage and high basal aldosterone secretion rates were the main

reasons for the failure of response seen in the acutely splanchnotomized dog. The failure to modify basal aldosterone secretion rate by severing the splanchnic nerves or by stimulating their central ends, and the return of the response to haemorrhage when the dogs were allowed to recover from the acute effects of splanchnotomy, demonstrated that neither afferent nor efferent activity of the splanchnic nerves were essential for the effect of haemorrhage on aldosterone secretion.

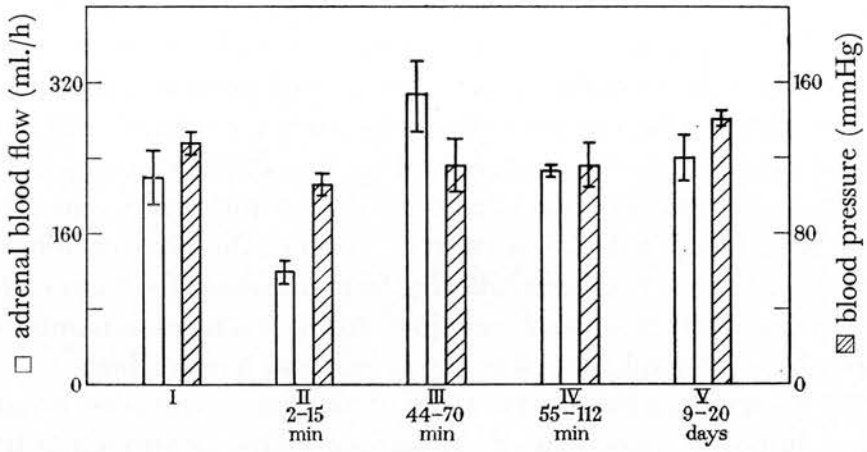

FIGURE 3. Effect of bilateral splanchnotomy on adrenal blood flow (□) and arterial pressure (▨). I and II: observations on 8 dogs, I, before, and II, after splanchnotomy (see table 11). III, IV and V: observations, after splanchnotomy, on three different groups of dogs (see tables 9, 10 and 12). Figures beneath columns indicate time after splanchnotomy at which adrenal blood collection was started.

VI. EFFECT OF VAGOTOMY ON THE RESPONSE TO HAEMORRHAGE

Farrell (1959) found that sectioning the vagus nerves in non-hypophysectomized, anaesthetized dogs caused wide fluctuations in the secretion rates of aldosterone and he assumed that vagal impulses affect aldosterone output. Mills, Casper & Bartter (1958) did not find significant changes in basal aldosterone secretion after vagotomy. However, the decrease in aldosterone secretion which follows the release of constriction of the inferior vena cava was not seen in vagotomized dogs. They concluded that the vagi form part of a reflex arc involved in decreasing aldosterone output.

In the course of the present work the effect of haemorrhage on aldosterone secretion was studied in vagotomized dogs.

Procedures and observations

In eight dogs both vagi were divided in the neck. In one group of six dogs the blood volume was increased initially by an infusion of about 18 ml./kg body weight of dog's blood or of 'Dextraven' (dextran, 6 g/100 ml. in 0·9 % NaCl). Adrenal blood was collected, then the dogs were bled (7 to 22 ml./kg body weight), and a second sample of blood was taken. Steroids were extracted from plasma only.

The mean aldosterone secretion (±S.E.) after volume expansion was $3{\cdot}6 \pm 1{\cdot}0 \mu g$ $(g\,adrenal)^{-1}\,h^{-1}$. It rose after haemorrhage in five dogs by 31 to 189 %. However, the haematocrit was slightly lower after haemorrhage and the proportion of the total blood

aldosterone estimated in the plasma higher in the second sample than in the first, thus exaggerating the actual rise in secretion. The experiments were, therefore, repeated in two dogs in which the steroids were extracted from whole blood. No initial expansion of the blood volume was carried out. Table 13 shows that both dogs responded to haemorrhage with a rise in aldosterone secretion.

TABLE 13. EFFECT OF HAEMORRHAGE ON ALDOSTERONE SECRETION IN VAGOTOMIZED DOGS

First adrenal blood sample (S_1) collected immediately after end of dissection, second sample (S_2) after haemorrhage. (Steroids extracted from whole blood, aldosterone figures not corrected for losses.)

dog no.	body weight (kg)	adrenal blood sample no.	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change) aldosterone	cortisol + corticosterone	adrenal blood flow (ml./h)	mean b.p. (mmHg)	blood withdrawn (ml./kg)	30 m-equiv. Na^+/day (no. of days)
135, male	11·0	S_1	7·8	2840	367	110	.	9
		S_2	11·7 +50	2793 −2	156	55	24	.
136, male	9·5	S_1	12·1	2651	112	130	.	2
		S_2	17·9 +48	2495 −6	64	78	18	.

Conclusions

There was no evidence to show that vagal activity directly influenced aldosterone secretion in dogs subjected to adrenal vein cannulation; the increase after haemorrhage was maintained, and so, apparently, was the fall after expansion of the blood volume, when secretion was $3{\cdot}6 \pm 1{\cdot}0 \mu$g (g adrenal)$^{-1}$ h^{-1}. This was similar to the figure of $3{\cdot}3 \pm 1{\cdot}0$ observed in a comparable group of dogs in which the vagi were intact (figure 2), and lower than the figures of $5{\cdot}3 \pm 1{\cdot}1$ found in the same group before expansion of the blood volume.

VII. ADRENAL MEDULLA AND ALDOSTERONE SECRETION

Some experiments were performed in order to find out whether the release of adrenaline and noradrenaline occurring during haemorrhage might participate in the mechanism which causes a rise in aldosterone secretion after haemorrhage.

Procedures and observations

In seven dogs the left and right splanchnic nerves were sectioned 53 to 137 min before collection of a control sample of adrenal blood. Intravenous infusion of L-adrenaline or L-noradrenaline-bitartrate (1μg min^{-1} (kg body wt)$^{-1}$) was then started 10 to 15 min before, and continued during, the collection of a second sample. A third blood sample was taken after an interval of 30 to 60 min.

The results are summarized in table 14. Of the three dogs given adrenaline, the one with the lowest initial secretion showed a significant rise in aldosterone production (dog 70), whereas no such rise occurred when 'resting' secretion was high (dog 71). The corresponding figures for dog 72 fell between the two extremes. Precisely the same happened when noradrenaline was infused: provided the secretion rate before the infusion was not over 6μg (g adrenal)$^{-1}$ h^{-1} (uncorrected), noradrenaline increased aldosterone production

(dogs 72, 74), but no rise was seen whenever initial secretion was higher (dogs 76, 77, 89). Glucocorticoid secretion was raised only once. Blood pressure changes were of the order of 20 %. These results show that medullary amines are able to accelerate aldosterone secretion in the splanchnotomized dog. It is probable that adrenomedullary secretion during haemorrhage contributes to the increase in aldosterone secretion, but such contribution is obviously not essential, as shown by the experiments in which the effect of haemorrhage persisted in splanchnotomized dogs.

TABLE 14. EFEFCT OF INTRAVENOUS INFUSIONS OF ADRENALINE AND NORADRENALINE ON CORTICOSTEROID SECRETION IN ACUTELY SPLANCHNOTOMIZED DOGS

First sample (S_1) collected immediately after the dissection, second (S_2) or fourth (S_4) sample started 10 to 15 min after the beginning of an infusion of L-adrenaline bitartrate (A) or L-noradrenaline bitartrate (N) which was continued during the whole collection period. Dose 1 μg base min^{-1} (kg body weight)$^{-1}$. (Steroids extracted from whole blood, aldosterone figures not corrected for losses.)

			adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change)			
dog no.	body wt. (kg)	adrenal blood sample no.	aldosterone	cortisol + corticosterone	adrenal blood flow (ml./h)	mean b.p. (mmHg)
70, male	12·6	S_1	0·9	917	89	138
		S_2 A	2·4 +167	1003 + 9	103	150
		S_3 32 min after S_2	1·4 − 42	852 −15	70	112
71, male	9·0	S_1	11·7	1075	175	95
		S_2 A	13·2 + 13	1288 +20	160	115
		S_3 30 min after S_2	14·3 + 8	1217 − 6	128	110
72, female	17·0	S_1	3·7	1070	194	104
		S_2 A	4·8 + 30	1207 +13	211	128
		S_3 30 min after S_2	5·5 + 15	1121 − 7	137	78
		S_4 N	8·1 + 47	1145 + 2	123	90
74, male	11·3	S_1	6·0	1424	192	112
		S_2 N	8·8 + 47	1500 + 5	179	140
		S_3 60 min after S_2	8·1 − 8	1565 + 4	108	100
76, female	17·5	S_1	8·4	1279	219	90
		S_2 N	9·0 + 7	1034 −19	120	77
77, male	17·5	S_1	7·5	1409	175	115
		S_2 N	7·6 + 1	1330 − 6	189	145
		S_3 35 min after S_2	7·8 + 3	1224 − 8	154	90
89, male	15·8	S_1	8·0	1151	172	135
		S_2 N	9·6 + 20	1545 +34	116	170
		S_3 61 min after S_2	11·2 + 17	1391 −10	92	129

VIII. OXYGEN LACK AND ALDOSTERONE SECRETION

Two sets of experiments were carried out in order to see whether the decrease in tissue oxygen tension participated in causing the rise in aldosterone secretion following acute haemorrhage.

Procedures and observations

In all animals a midline incision was made and the left adrenal vein cannulated. Steroids were extracted from whole blood and the aldosterone figures not corrected for losses.

Decrease in the oxygen content of the inspired air

In the first group of dogs anoxia was produced by decreasing the oxygen supply to the lungs. Once, mechanical obstruction of the trachea was used; in the other dogs artificial respiration was applied throughout the experiment, and the composition of the inspired air changed; a control sample of adrenal blood was taken while the dog was breathing air, then the inlet tube of the respiration pump was connected to a Douglas bag filled with a mixture of nitrogen and oxygen. The percentage of O_2 was varied between 10 and 17 %. The dogs inhaled this mixture for 4 or 5 min before, and during the entire collection period of a second blood sample. Then breathing was returned to room air and a few minutes later a third blood sample collected.

The results are listed in table 15. In most animals aldosterone secretion changed but little during the period of anoxia; there was, however, a large fall in both aldosterone and glucocorticoid secretion in dog 91 in which asphyxia was more severe than in the other dogs. In this experiment the oxygen supply to the adrenal must have been the limiting factor for hormone synthesis.

When breathing of normal air was resumed after anoxia, aldosterone secretion rose in eight of the nine dogs, 5 times significantly. Perhaps this rise represented an overshoot resulting from a hormone 'debt' incurred during the preceding period when hormone synthesis was restricted by the oxygen supply. There was little change in glucocorticoid secretion except for the one instance when it was much reduced during the anoxic period; neither were there large effects on adrenal blood flow or blood pressure. Mayer waves did not appear as a result of anoxia; in two of the experiments they were present throughout.

Though the nature of the influence of anoxia on secretion of aldosterone is not clear from these experiments, the results suggest that there are such influences, which become manifest at the time when a period of oxygen lack is superseded by normal oxygen supply.

Reduction of the number of circulating erythrocytes

In a second set of experiments tissue anoxia was produced by removal of red cells without changing the blood volume. This was done by exchange-transfusion with dextran ('Dextraven', as before) or dog's plasma (kept for 1 to 2 days at +4 °C, filtered and warmed immediately before use). After the collection of a control sample of adrenal blood, 40 ml./kg body wt. of either fluid was infused into a femoral vein and an equal volume of blood simultaneously withdrawn from a femoral artery. The exchange was completed in 10 to 15 min and was followed by a second collection of adrenal vein blood. The blood taken from the adrenal vein was replaced by blood with low haematocrit in order to keep the proportion of red cells low. In seven dogs the splanchnic nerves of both sides were sectioned 1 h before collecting the first sample; this was done in order to block hypothetical chemoreceptor impulses which might ascend in the splanchnic nerves.

The results are summarized in figures 4 to 6 and table 16. Figure 4*a* shows aldosterone secretion rates observed on a group of dogs in which the daily Na^+ intake was 30 m-equiv. The five dogs (left panel, reactors) which had been on the low Na^+ diet for a very short period had low initial aldosterone figures and responded to transfusion with plasma by a rise in secretion. None of the 15 dogs which had been left on this diet for a longer period

responded. Many, but not all, animals had a high initial rate of aldosterone secretion (figure 4*a*, right panel).

Figure 5 shows the observations obtained on six dogs in which dextran was exchanged against blood. The dogs had been newly admitted. Only one dog responded with a rise, and this was the animal with the lowest initial secretion rate.

The picture (figure 6) for a group of seven dogs which were splanchnotomized immediately before the experiment is not very different. The dogs were on the low Na^+ diet

TABLE 15. EFFECT OF REDUCING THE OXYGEN CONTENT OF THE INSPIRED AIR ON ALDOSTERONE SECRETION

Respiratory rate kept at 20/min. Dietary sodium intake unknown. Midline incision. (Steroids extracted from whole blood, aldosterone figures not corrected for losses.)

dog no.	body wt. (kg)	adrenal blood sample no.	conditions	duration of collection periods (min)	volume delivered per stroke/ kg body weight (ml.)	% O_2 in mixture inhaled	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change) aldosterone	cortisol + corticosterone	adrenal blood flow (ml./h)	mean b.p. (mmHg)	Mayer waves
88, female	10	S_1	control				5·4	934	255	153	–
		S_2	partial occlusion of trachea started 9 min before and continued during collection	40	.	.	6·7 + 24 + 25	725 − 22 + 26	237	150	–
		S_3	started immed. after airways freed				8·4	911	186	143	–
90, male	10·8	S_1	control				5·7	1550	511	124	–
		S_2	during anoxia	25	18·5	10	5·2 − 9	1494 − 4	684	130	–
		S_3	started 5 min after return to room air				6·3 + 21	1714 + 15	432	126	–
91, male	19·5	S_1	control				7·6	1808	264	167	–
		S_2	during anoxia	30	15·4	10	1·9 − 75	1188 − 34	306	135	–
		S_3	started 7 min after return to room air				8·0 + 321	1860 + 57	256	150	–
92, female	18·0	S_1	control				7·1	1090	552	120	–
		S_2	during anoxia	20	16·7	15	7·7 + 8	1096 + 0	651	127	–
		S_3	started 10 min after return to room air				10·8 + 40	1120 + 2	561	118	–
93, male	10·4	S_1	control				11·0	1128	154	158	–
		S_2	during anoxia	30	14·4	15	7·8 − 29	1148 + 2	148	172	–
		S_3	started 7 min after return to room air				14·6 + 87	1121 − 2	120	185	–
94, male	12·6	S_1	control				5·1	1221	248	165	–
		S_2	during anoxia	30	23·4	17	7·1 + 39	1192 − 2	276	152	–
		S_3	started 10 min after return to room air				5·9 − 17	1133 − 5	226	96	–
95, male	9·0	S_1	control				6·9	1653	126	103	+
		S_2	during anoxia	40	22·2	17	5·6 − 19	1647 0	126	110	+
		S_3	started 10 min after return to room air				8·4 + 50	1882 + 14	93	135	+
96, male	13·5	S_1	control				9·7	1423	372	159	+
		S_2	during anoxia	25	14·8	16	8·2 − 15	1492 + 5	372	155	+
		S_3	started 10 min after return to room air				10·0 + 22	1319 − 12	293	152	+
97, female	6·8	S_1	control				5·0	994	144	148	–
		S_2	during anoxia	35	18·8	16	5·5 + 10	1091 + 10	144	175	–
		S_3	started 10 min after return to room air				9·3 + 69	1104 + 1	114	168	–
mean ± S.E.		S_1	control				7·1 ± 0·7	1311 ± 103	292 ± 52	144 ± 8	
		S_2	during anoxia				6·2 ± 0·6	1230 ± 92	327 ± 70	145 ± 7	
		S_3	after anoxia				9·1 ± 0·9	1352 ± 123	253 ± 52	141 ± 9	

for 1 or 2 days only, too short a period to cause sodium deficiency and increase aldosterone production, except for one non-reactor which was given this diet for 6 days. The high initial aldosterone secretion seen in four of the five non-reactors is most probably due to the stressing effect of the operation (see section on acute splanchnotomies). In fact, it is remarkable that two dogs 'reacted', and that one of them did so in spite of a high initial output of aldosterone.

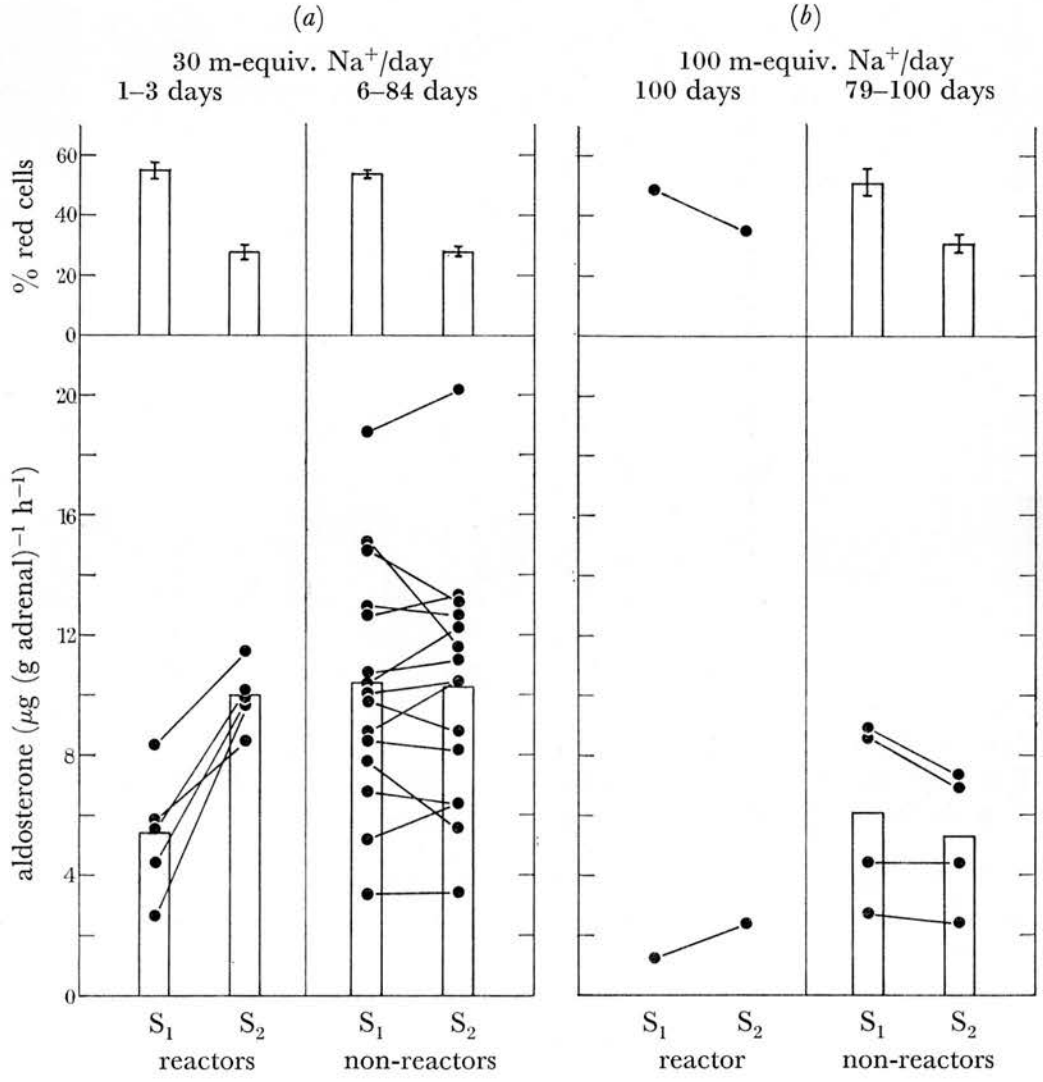

FIGURE 4. (*a*) dogs on low, (*b*) dogs on high dietary sodium intake. Aldosterone secretion before (S_1) and after (S_2) decreasing the haematocrit by an exchange transfusion with dog's plasma ($39 \pm 0{\cdot}8$ ml./kg). (Steroids extracted from whole blood, aldosterone figures not corrected for losses.) ●, individual experiments; Columns, means ± S.E.

Figure 4*b* represents the results in five dogs which had been on a high sodium intake for an excessively long period. This procedure considerably reduced the initial rate of aldosterone secretion, yet a response to the loss of red cells occurred only once, and, precisely as in the group represented in figure 5, the reactor was the dog with the smallest resting secretion. Even so, the response was much smaller than in the dogs of figure 4*a*, which had

not been given extra salt, and one cannot escape the suspicion that the chronic depression of aldosterone secretion by sodium loading had also reduced the reactivity of the aldosterone synthesizing mechanism. The findings on dog 275 in the series of chronically splanchnotomized dogs had previously suggested a similar conclusion.

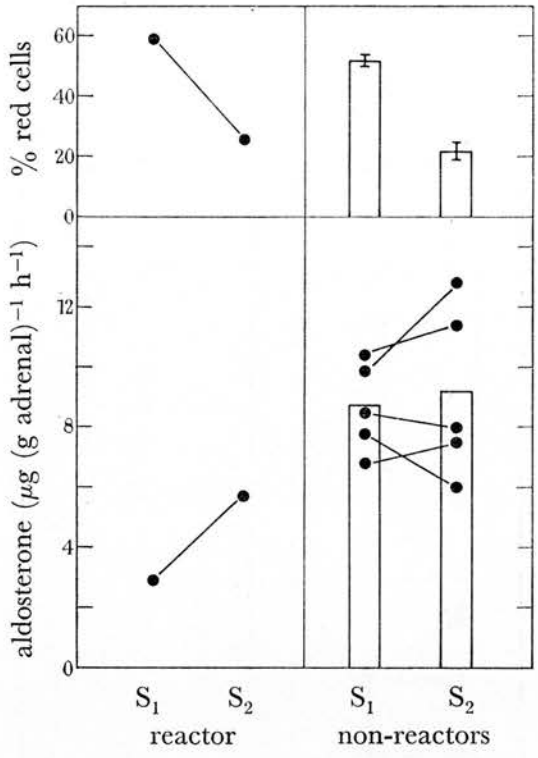

FIGURE 5. Aldosterone secretion before (S_1) and after (S_2) decreasing the haematocrit by exchange transfusion with dextran ($46 \pm 3{\cdot}1$ ml./kg). Dogs newly admitted and dietary sodium intake therefore unknown. (Steroids extracted from whole blood, aldosterone figures not corrected for losses.) ●, individual experiments; Columns, means ± S.E.

The means of the glucocorticoid secretion rates, of adrenal blood flow and arterial blood pressure are given in table 16. Glucocorticoid secretion was not affected by the removal of red cells. The largest amounts of glucocorticoids were secreted by the dogs which had been on a high sodium intake for a prolonged period. The plasma was usually well tolerated; at the beginning of the exchange there was frequently a slight fall in blood pressure followed as a rule by a rise of 20 to 40 mmHg above the original value and then by a gradual return to normal. Mayer waves occurred only in two dogs during collection of the second sample. In three dogs Mayer waves were present throughout the experiment.

Conclusions

The results suggest that a fall in the oxygen tension in the tissues can contribute to the rise in aldosterone secretion which follows upon haemorrhage. However, in the experiments on exchange-transfusion the proportion of 'reacting' dogs was small when compared with the percentage normally seen after haemorrhage. This is not surprising when the experiments on acutely splanchnotomized dogs and on dogs kept for an excessively long time on a high Na^+ intake (figures 6 and 4*b*) are considered; these experiments were car-

ried out before it was known that these procedures create adverse conditions for a further rise in aldosterone output—splanchnotomy, by raising initial aldosterone secretion rate, excessive sodium intake by apparently depressing the reactivity of the aldosterone producing cells. When, however, the large number of non-reactors in figure 4*a* is considered, it is

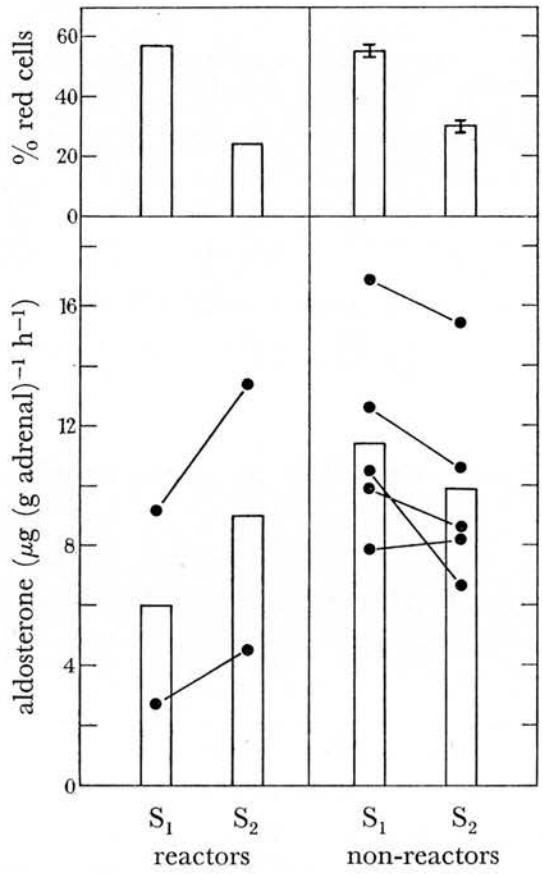

FIGURE 6. Aldosterone secretion of acutely splanchnotomized dogs before (S_1) and after (S_2) decreasing the haematocrit by an exchange transfusion with dog's plasma ($39 \pm 1{\cdot}7$ ml./kg). Daily sodium intake 30 m-equiv. (reactors for 1 day, non-reactors for 1 to 6 days). (Steroids extracted from whole blood, aldosterone figures not corrected for losses.) ●, individual experiments; Columns, means ± S.E.

difficult to escape the conclusion that loss of red cells is a less potent stimulus of aldosterone production than haemorrhage and can therefore play only a minor role in eliciting the response to haemorrhage. On the assumption that the increase in aldosterone secretion seen after exchange transfusion is elicited by a reflex, involving chemoreceptor activity, the results on splanchnotomized dogs suggest that such activity is not transmitted to the centres by the splanchnic nerves.

DISCUSSION

The experiments described in this paper were done on dogs in which pituitary and kidneys were intact. It is obvious from the results that aldosterone secretion is as subject to being increased by a multitude of noxious conditions or 'stresses' as is glucocorticoid secretion. Theconcept held 10 years ago that *ACTH* affects production of aldosterone but little

TABLE 16. OBSERVATIONS ON GLUCOCORTICOID SECRETION, ADRENAL BLOOD FLOW AND MEAN ARTERIAL PRESSURE

First sample (S_1) before, second sample (S_2) after an exchange transfusion with dextran or dogs' plasma (40 ml./kg). Means ± standard errors. A_1: arterial blood sample taken before S_1; A_2: arterial blood sample taken after S_2.

dog nos.	adrenal blood sample	cortisol + corticosterone (μg (g adrenal)$^{-1}$ h^{-1})	adrenal blood flow (ml./h)	mean b.p. (mmHg)	arterial blood sample	Ht (% red cells)	plasma conc. (m-equiv./l.) Na^+	plasma conc. (m-equiv./l.) K^+
GROUP I. BLOOD-PLASMA EXCHANGE, SPLANCHNIC NERVES INTACT (ALDOSTERONE SEE FIGS. 4 (*a*) AND 4 (*b*))								
A. Reactors (30 m-equiv. Na^+/day for 1 to 3 days)								
112, 113, 114, 122, 123	S_1	1411 ± 190	207 ± 36	132 ± 9	A_1	55 ± 2·1	.	.
	S_2	1483 ± 181	299 ± 54	126 ± 11	A_2	28 ± 1·9	.	.
B. Non-reactors (30 m-equiv. Na^+/day for 6–84 days)								
110, 115, 117, 119, 126, 141, 144, 145, 147, 148, 149, 150, 151, 152, 154	S_1	1568 ± 82	256 ± 24	125 ± 7	A_1	54 ± 1·0	148 ± 2	3·7 ± 0·1
	S_2	1535 ± 81	289 ± 25	121 ± 8	A_2	28 ± 0·8	148 ± 1	4·1 ± 0·1
C. Reactor (100 m-equiv. Na^+/day for 100 days)								
230	S_1	1893	278	168	A_1	49	156	3·0
	S_2	1899	276	164	A_2	35	145	3·2
D. Non-reactors (100 m-equiv. Na^+/day for 79–100 days)								
221, 227, 228, 231	S_1	1827 ± 133	215 ± 26	146 ± 2	A_1	51 ± 5	148 ± 2	3·6 ± 0·2
	S_2	1944 ± 337	216 ± 42	145 ± 3	A_2	31 ± 3	144 ± 2	3·4 ± 0·1
GROUP II. BLOOD-DEXTRAN EXCHANGE, SPLANCHNIC NERVES INTACT, NEWLY ADMITTED (ALDOSTERONE SEE FIGURE 5)								
A. Reactor								
98	S_1	1017	154	148	A_1	59	.	.
	S_2	1057	185	130	A_2	26	.	.
B. Non-reactors								
100, 101, 103, 105, 106	S_1	1040 ± 70	308 ± 34	142 ± 12	A_1	52 ± 1·2	.	.
	S_2	1371 ± 104	306 ± 42	121 ± 15	A_2	22 ± 2·3	.	.
GROUP III. ACUTELY SPLANCHNOTOMIZED DOGS; BLOOD-PLASMA EXCHANGE (ALDOSTERONE SEE FIGURE 6)								
A. Reactors (30 m-equiv. Na^+/day for 1 day)								
118, 128	S_1	1523, 1200	81, 123	95, 114	A_1	52, 62	.	.
	S_2	1408, 1098	80, 158	63, 104	A_2	25, 24	.	.
B. Non-reactors (30 m-equiv. Na^+/day for 1–6 days)								
116, 121, 124, 127, 129	S_1	1600 ± 66	146 ± 31	100 ± 10	A_1	55 ± 2	.	.
	S_2	1578 ± 58	148 ± 42	92 ± 14	A_2	30 ± 2	.	.

has since been revised (Davis, Carpenter, Ayers & Bahn 1960; Mulrow, Ganong, Kuljian & Boryczka 1961; Holzbauer 1964), and the ready rise in aldosterone secretion during stress should, in part, be due to the stimulating effect of *ACTH*. In the meantime detailed knowledge has also accumulated on the effect of angiotensin (Carpenter, Davis & Ayers 1961*b*; Mulrow *et al.* 1961; Slater, Barbour, Henderson, Casper & Bartter 1963) on aldosterone secretion in dogs. Renin, too, is probably released during surgery so that the 'basal'

secretion rate of aldosterone will also depend on the amount of the angiotensin formed by it in the circulating blood.

In spite of the fact that aldosterone secretion was elevated as a result of adrenal vein cannulation, it was possible to elicit further rises in aldosterone secretion by haemorrhage in about one half of the animals not subjected to further surgery. This relatively small percentage of 'reactors' necessitated the use of large groups of dogs in order to establish the importance of any factor in the acceleration of aldosterone secretion after haemorrhage. The greatest difficulty was encountered when, in order to exclude certain structures of organs as participants in the effect, procedures were required which so raised aldosterone production before haemorrhage that no further rise could be expected. Some of the difficulties were avoided by preliminary aseptic operations from which the dog was allowed to recover before the experiment was carried out. In some experiments, incipient circulatory failure was associated with high initial secretion rate of aldosterone. In such dogs, the circulation sometimes deteriorated to the point of shock when the dog was bled; in this condition aldosterone secretion usually fell to low levels.

The experiments have established that the presence of the following structures was not required for the response to haemorrhage to occur: the vagi, the splanchnic nerves, the baroreceptors of carotid sinus and thyro-carotid junctions, the chemoreceptors of the carotid sinus, the liver, spleen and gastrointestinal tract. Provided the initial secretion was low enough, small rises in aldosterone secretion were seen after infusion of adrenaline and noradrenaline in the splanchnotomized animal, after exchange transfusions involving a loss of 50 % of the dog's red cells, and as an after-effect of a period of breathing a mixture of low oxygen content. It is likely that these rises were brought about by a release of *ACTH*. The fact that the effects were small shows that anoxia alone is a less potent stimulus of aldosterone secretion than is blood loss, and suggests that the circulatory effect of haemorrhage plays an important role in eliciting the response, the mediating agents presumably including both *ACTH* and renin.

Two obvious questions are posed by this work: are we to assume that, in the anaesthetized animal subjected to surgical stress, haemorrhage releases enough additional *ACTH* and renin to account for the further rise in aldosterone secretion, or is there yet another 'aldosterone-releasing factor' involved? Is the release of *ACTH* or that of renin more important when a dog is bled under the conditions of these experiments?

An attempt at answering these questions will be made in the next paper dealing mainly with experiments on hypophysectomized and nephrectomized dogs.

Our thanks are due to Benger Laboratories for a gift of Dextraven, to Dr C. D. Falconer (Ciba Laboratories) for a sample of aldosterone and to the Endocrinology Study Section, U.S. Department of Health for a gift of 7-^{3}H-aldosterone. To the U.S. Department of Health, Education and Welfare we would like to express our gratitude for a grant to buy a Tri-Carb Scintillation Spectrometer.

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INVESTIGATIONS INTO THE CAUSES OF THE RISE IN ALDOSTERONE SECRETION DURING HAEMORRHAGE. PART II

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CONTENTS

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The role of *ACTH* and of renin as mediators of the stimulating effect of haemorrhage on aldosterone secretion was investigated. The following experiments showed that release of *ACTH* is not indispensible for the effect: in non-hypophysectomized dogs with intact kidneys, in which the blood *ACTH* concentration was artificially raised by infusing *ACTH*, there was still a rise in aldosterone production after blood loss. Hypophysectomy did not abolish or reduce the response, in fact, it increased its frequency of occurrence in dogs in which steroid synthesis was maintained at a submaximal level by a constant infusion of *ACTH*.

Another group of studies demonstrated that the release of renin is also not a necessary condition for the rise in aldosterone production after bleeding; dogs in which both kidneys had been removed, but the pituitary left intact, responded to bleeding by a rise in aldosterone secretion of the same magnitude as normal dogs. However, in the simultaneous absence of kidneys and pituitary gland aldosterone production did not rise after bleeding although the basic conditions for synthesis of steroids were provided by a constant infusion of *ACTH*. On the contrary, severe falls in steroid secretion rates were the rule.

These falls were attributed to the fact that hypophysectomized-nephrectomized dogs often reacted to blood loss with collapse of the circulation, and it was possible to argue that this collapse, and not the absence of kidney and pituitary, might have prevented the rise in aldosterone secretion. Attempts were therefore made to improve the circulation by supplying pressor substances known to be released in haemorrhage: of these noradrenaline did not improve the tolerance to haemorrhage, angiotensin improved it only very slightly, but prolonged infusions of extracts of posterior lobe restored it nearly to normal. In some of these experiments, the post-haemorrhage fall in aldosterone secretion was also prevented, but a rise was never seen.

Aldosterone secretion of the hypophysectomized-nephrectomized dog was stimulated by infusion of large volumes of donor blood obtained from dogs with intact kidneys which presumably contains renin, but not of blood from nephrectomized donors.

No evidence was obtained for the existence of agents other than *ACTH* and angiotensin as mediators of the stimulating effect of haemorrhage on aldosterone secretion. Furthermore, these two agents could fully replace each other, as shown by the finding that the effect of haemorrhage was not diminished by either nephrectomy or hypophysectomy alone.

INTRODUCTION

This paper examines the role played by the pituitary gland and the kidneys in the sequence of events which leads to a rise in aldosterone secretion following haemorrhage. Effects of *ACTH* and of angiotensin on aldosterone secretion are now well established. The questions dealt with are whether the two substances can replace each other or whether one of them can be shown to be more important than the other in the adrenal response to haemorrhage; furthermore, whether a response can still be obtained when pituitary and kidneys are removed.

METHODS

Most operative procedures and the chemical methods used in these experiments have been outlined in the preceding paper. Steroids were extracted from whole blood in all experiments. Two batches of *ACTH* were used: for the experiments in chapter I 'Cortrophin' Organon, (corticotrophin B.P.) for intravenous use, carboxy-cellulose-purified; for all remaining experiments, 'Cortrophin' Organon, of porcine origin, for intramuscular use; this batch was the same as that used by Holzbauer (1964). The *ACTH* was administered by a constant-infusion pump through a fine needle inserted into an unobstructed jugular vein. All solutions were acidified with HCl.

The pituitary gland was removed through the roof of the mouth at least 2 h before starting the collection of samples of adrenal blood. Completeness of the operation was checked by inspection of the sella turcica and the base of the brain at the end of the experiment.

During extirpation the kidneys were handled as little as possible and, from dog 301 onwards, the renal arteries and veins were ligated simultaneously in order to minimize the escape of renin into the circulation.

RESULTS

I. Aldosterone secretion before and after haemorrhage in dogs with intact pituitary given infusions of *ACTH*

The object of these experiments was to try and cancel out the effect of any *ACTH* released as a result of bleeding by artificially producing a high constant background of blood *ACTH*. If release of *ACTH* was indispensable for the rise in aldosterone secretion after haemorrhage, this procedure should reduce or abolish the response. Infusion was made into the jugular vein and either started before adrenal blood was taken or after collection of control samples. Unless otherwise stated it was continued to the end of the experiment.

Procedures and observations

Dogs bled once

One or two control samples of adrenal venous blood were collected before, and one after the start of a continuous infusion of *ACTH*; the dogs were then bled and a further sample taken. In preliminary experiments (table 1) a wide range of doses of *ACTH* was explored; in the remaining dogs the mean amount infused (0·35 m-u. min^{-1} (kg body wt.)$^{-1}$) was slightly higher than the lower concentrations used before. Only the aldosterone figures of this last group are corrected for losses and the data are therefore listed separately (table 2).

The results were the same for all doses of *ACTH*. None of the 15 dogs responded to the *ACTH* infusion with a significant rise in the secretion rates of cortisol and corticosterone, but in three dogs (204, 208, 251) aldosterone secretion rose by 40, 32 and 73 % respectively; five dogs (201, 204, 205, 207, 253) responded to haemorrhage with rises in aldosterone. Three of the four non-reactors of table 1 (dogs 198, 208, 229) had a high aldosterone secretion immediately before the haemorrhage; lack of response in the fourth dog (202) is probably accounted for by adrenal damage (suggested by the simultaneous fall, after bleeding, in secretion of aldosterone and glucocorticoids). Among the seven dogs of table 2, there was only one, 253, in which aldosterone secretion rate was low before bleeding, and this was the only dog that responded.

Dogs bled twice

The preceding experiments showed that corticosterone and cortisol only rarely rose in dogs when they were given infusions of *ACTH* ranging from 0·09 to 1·7 m-u. min^{-1} kg^{-1}. This suggested that the conditions of the experiment released enough *ACTH* to produce maximal secretion of glucocorticoids, though not necessarily of aldosterone. Among the infused dogs reactors were fewer than normal. To try and overcome the difficulty caused by individual variations in the response, experiments were carried out in which the effect of haemorrhage was examined in the same dog before and during infusion of *ACTH*, the blood volume being restored between the two halves of the experiment.

Samples of adrenal blood were collected before and after bleeding, the shed blood was re-infused, and either 10 or 60 min later another sample was taken. Then an infusion of *ACTH* (0·2 to 0·35 m-u. min^{-1} kg^{-1} body weight) was started, and adrenal blood collected

TABLE 1. EFFECT OF *ACTH*-INFUSION IN DOGS WITH INTACT PITUITARY GLAND ON CORTICOSTEROID SECRETION BEFORE AND AFTER HAEMORRHAGE

First adrenal blood sample collected 30 min after end of dissection. Collection time usually 25 to 30 min, but 15 min in dog 205. One or two samples (S_0 and S_1) before, the remaining samples during constant infusion of ACTH. S_2 before, S_3 after haemorrhage. Na^+ intake 100 m-equiv./day for 9 to 31 days. (Aldosterone figures not corrected for losses.)

dog no.	body wt. (kg)	adrenal blood sample no.	dose of *ACTH* (m-u. $min^{-1}\,kg^{-1}$)	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change): aldosterone	cortisol + corticosterone	adrenal blood flow (ml./h)	mean b.p. (mmHg)	Mayer waves	Ht (% red cells)	blood withdrawn before S_3 (ml./kg)
Group I (midline incision)										
198,* male	11·7	S_1	0	5·1	999	91	155	+	61	.
		S_2	1·6	5·8 +14	1019 + 2	120	155	+	62	.
		S_3	1·6	4·5 −22	954 − 6	96	80	+	58	10
201, male	10·4	S_1	0	3·3	2050	166	125	−	52	.
		S_2	1·6	3·8 +15	2035 − 1	149	142	−	50	.
		S_3	1·6	6·5 +71	2190 + 8	115	70	+	49	20
202, male	10·8	S_1	0	4·2	1660	144	156	−	56	.
		S_2	1·7	4·5 + 7	1718 + 3	156	162	+	55	.
		S_3	1·7	2·3 −49	1307 −24	101	120	+	55	20
mean ± S.E.		S_1	0	4·2 ± 0·5	1570 ± 307	134 ± 22	145 ± 10		56 ± 2·6	.
		S_2	1·63	4·7 ± 0·6	1591 ± 300	142 ± 11	153 ± 6		56 ± 3·4	.
		S_3	1·63	4·4 ± 1·2	1484 ± 368	104 ± 6	90 ± 15		54 ± 2·6	17 ± 3
Group II (right flank incision)										
204, female	9·0	S_1	0	1·5	1335	92	137	−	43	.
		S_2	0·2	2·1 +40	1371 + 3	88	137	−	43	.
		S_3	0·2	3·1 +48	1264 − 9	48	120	+	46	20
205, female	19·3	S_1	0	4·1	1527	324	170	−	43	.
		S_2	0·09	3·3 −20	1420 − 7	244	170	−	41	.
		S_3	0·09	6·4 +94	1869 +22	192	166	−	43	20
207, male	11·6	S_1	0	7·3	1807	187	192	+	57	.
		S_2	0·3	4·9 −33	1975 + 9	175	180	−	55	.
		S_3	0·3	7·0 +43	1789 − 9	156	80	+	50	21
208, female	11·2	S_1	0	3·8	1473	314	175	−	60	.
		S_2	0·3	5·0 +32	1581 + 7	343	180	+	58	.
		S_3	0·3	5·6 +12	1808 +14	295	145	+	58	20
229, male	13·5	S_0	0	6·1	2237	262	162	−	49	.
		S_1	0	6·4 + 5	2230 0	235	165	−	49	.
		S_2	0·3	8·3 +30	2435 + 9	240	168	−	51	.
		S_3	0·3	8·5 + 2	2090 −14	144	141	+	48	18
mean ± S.E.		S_1	0	4·6 ± 1·0	1674 ± 159	230 ± 43	168 ± 9	.	50 ± 3	.
		S_2	0·24	4·7 ± 1·0	1756 ± 200	218 ± 42	167 ± 8	.	50 ± 3	.
		S_3	0·24	6·1 ± 0·9	1764 ± 136	167 ± 40	130 ± 15	.	49 ± 3	20 ± 0·5

* Enteritis.

before and after bleeding. Figure 1 shows the results. Three of the seven dogs reacted to the first bleeding by increases in aldosterone secretion ranging from 44 to 135 %, but re-infusion of the lost blood did not restore the initial low aldosterone secretion. The subsequent infusion of *ACTH* did not raise either aldosterone or glucocorticoid secretion.

TABLE 2. EFFECT OF *ACTH*-INFUSIONS IN DOGS WITH INTACT PITUITARY GLAND ON CORTICOSTEROID SECRETION BEFORE AND AFTER HAEMORRHAGE

First adrenal blood sample collected 30 min after end of dissection. Collection time 25 to 30 min. One or two samples (S_0 and S_1) before, the remaining samples during constant infusion of *ACTH*. S_2 before, S_3 after haemorrhage. Na^+ intake 100 m-equiv./day for 7 to 42 days. (Flank incision, aldosterone figures corrected for losses.)

dog no.	body wt. (kg)	adrenal blood sample no.	dose of *ACTH* (m-u. min^{-1} kg^{-1})	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change) aldosterone	cortisol + corticosterone	adrenal blood flow (ml./h)	mean b.p. (mmHg)	Mayer waves	Ht (% red cells)	blood withdrawn before S_3 (ml./kg)
234, male	15·4	S_0	0	13·0	1551	226	150	+	54	.
		S_1	0	15·9 +22	1522 − 2	197	156	+	50	.
		S_2	0·3	19·3 +21	1160 −24	173	158	−	49	.
		S_3	0·3	22·3 +16	1351 +16	82	78	+	50	18
235, male	15·3	S_0	0	22·6	1832	118	164	−	50	.
		S_1	0	23·4 + 4	1761 − 4	118	165	−	50	.
		S_2	0·3	24·0 + 3	1638 − 7	115	160	−	49	.
		S_3	0·3	28·4 +18	1426 −13	67	80	+	51	16
236, male	14·4	S_0	0	20·3	2144	246	133	−	53	.
		S_1	0	18·4 − 9	1646 −23	230	140	−	.	.
		S_2	0·3	21·0 +14	1214 −26	177	145	−	53	.
		S_3	0·3	21·7 + 3	1712 +41	99	75	+	50	18
250, male	11·7	S_1	0	9·7	1520	240	173	−	53	.
		S_2	0·4	10·4 + 7	1731 +14	193	170	−	51	.
		S_3	0·4	11·1 + 7	1996 +15	156	114	+	53	20
251, male	11·6	S_1	0	7·1	2079	286	155	−	40	.
		S_2	0·4	12·3 +73	2090 0	274	160	−	43	.
		S_3	0·4	13·3 + 8	1525 −27	143	82	+	44	16
252, male	14·4	S_1	0	8·2	1221	264	182	−	67	.
		S_2	0·3	9·8 +15	1500 +23	240	182	−	65	.
		S_3	0·3	8·9 − 5	1116 −26	274	136	+	61	20
253, female	9·4	S_1	0	5·7	1673	98	150	−	47	.
		S_2	0·5	4·9 −14	1317 −21	84	140	−	49	.
		S_3	0·5	10·5 +114	1495 +14	126	95	+	44	20
mean ± S.E.		S_0	0	18·6 ± 2·9	1842 ± 171	197 ± 40	149 ± 9	.	52 ± 1·2	.
		S_1	0	12·6 ± 2·5	1632 ± 99	205 ± 27	160 ± 5	.	51 ± 3·6	.
		S_2	0·35	14·5 ± 2·6	1521 ± 124	179 ± 25	159 ± 5	.	51 ± 2·6	.
		S_3	0·35	16·6 ± 2·8	1517 ± 105	135 ± 26	94 ± 9	.	50 ± 2·2	18 ± 0·7

Of the six dogs in which adrenal blood was obtained after a second haemorrhage, three responded with a rise in aldosterone secretion, which was significant in two animals. The reactors were not the ones which had reacted initially, but those with the lowest secretion rate just before haemorrhage (see figure 1). The need for considering individual experiments on their own merits is illustrated by dog 242 (figure 1) in which the failure to respond to the first haemorrhage may have been due to an elevated initial secretion rate, but may equally have been the result of an unusually high haematocrit, which was 79 % at the time of the first bleeding. Under these circumstances loss of blood must have had a beneficial effect on circulation and failed to elicit compensatory mechanisms. Before the second haemorrhage the haematocrit was 66 %, and now a response was obtained.

The dogs which were bled twice were difficult to keep in good condition to the end of the

experiments. In order to have conditions optimal while *ACTH* was being infused, the procedure was therefore reversed in three dogs: *ACTH* was infused before and during the first haemorrhage and discontinued before restoration of the blood volume. An hour later a second pair of samples was taken, one before and one after bleeding. The result was clear-cut (figure 2). All dogs responded to the first bleeding, in spite of the fact that *ACTH* was

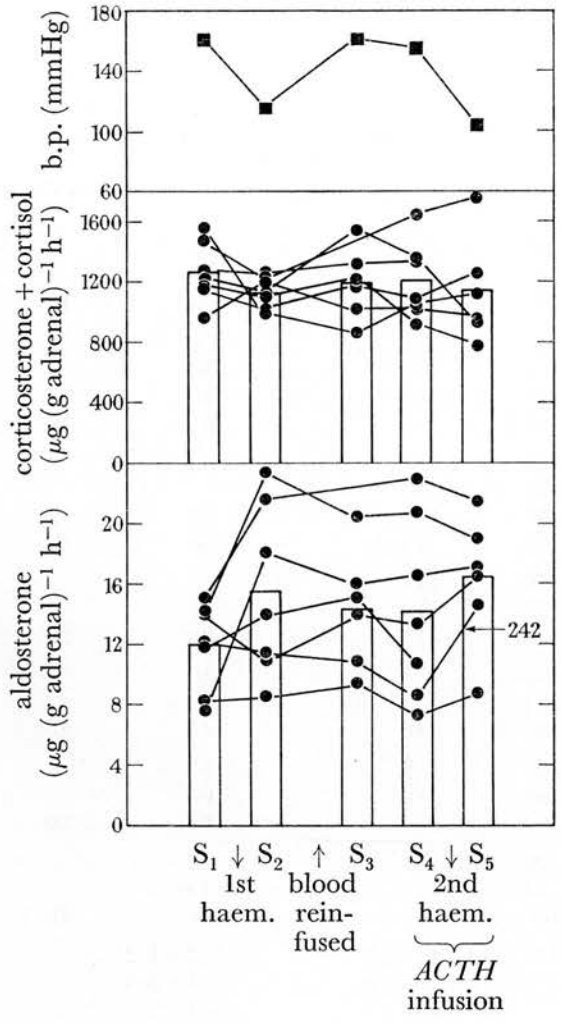

FIGURE 1

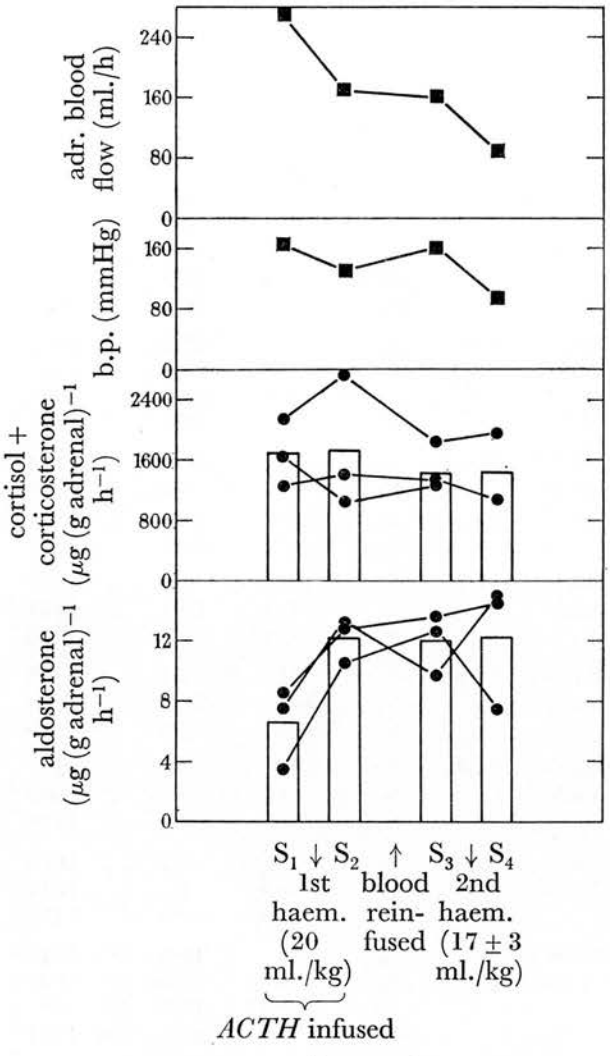

FIGURE 2

FIGURE 1. Effect of *ACTH* infusion on the aldosterone response to blood loss in dogs with intact pituitary. Adrenal blood samples collected for 15 to 30 min: S_1 before, S_2 after first haemorrhage (20 ± 0 ml./kg); S_3 10 to 60 min after restoration of the initial blood volume; S_4 5 min after the start of an *ACTH* infusion (0·2 to 0·35 m-u. min^{-1} (body kg wt)$^{-1}$); S_5 after second haemorrhage (18 ± 1 ml./kg). *ACTH* infusion stopped 5 min before end of S_5. Daily sodium intake 100 m-equiv. for more than 7 days. Flank incision, aldosterone figures corrected for losses. ●, Individual observations; columns and ■, means.

FIGURE 2. Effect of *ACTH* infusion on the aldosterone response to blood loss in dogs with intact pituitary. Experiments similar to those in figure 1, but *ACTH* infusion (0·4 to 0·5 m-u. min^{-1} kg^{-1} body weight) during first haemorrhage, started 5 min before S_1 and stopped 5 min before end of S_2. Daily sodium intake 100 m-equiv. for 2 to 14 days. Flank incision, aldosterone figures corrected for losses. ●, Individual observations; columns and ■, means.

being infused, but only one dog showed an increased output of aldosterone at the second haemorrhage. Characteristically, it was the dog with the lowest secretion during the second control period.

Comparison of infusion of freshly shed and day-old donor blood

In the experiments of the two previous groups (figures 1, 2) it was found nearly impossible to depress aldosterone secretion to pre-haemorrhage rates by re-infusing the dog's own blood. This raised the question whether freshly drawn blood contained some active 'glomerulotrophin'. In donor-blood which had been standing for 24 h or longer, such substances might have been destroyed, as would be the fate of any *ACTH* present (Nelson & Hume 1955). Experiments were therefore carried out in order to see whether infusion of 'stale' blood would be better suited than that of fresh blood to decrease aldosterone secretion which had been raised by blood loss. With the intention of starting from a high rate of aldosterone secretion, the animal was bled 20 ml./kg while *ACTH* (0·2 m-u. min^{-1} kg^{-1}) was being infused. Shortly after the haemorrhage, a first adrenal blood sample was collected, while the infusion of *ACTH* was continued. Then the initial blood volume was restored by infusing 1-day-old donor blood. About 5 or 45 min later, a second adrenal blood sample was taken, the dog then bled a second time, and a third sample collected. Details and results are found in table 3. In two dogs (258, 259) aldosterone secretion fell after re-infusion of the lost blood; the effect was rapid, since the intervals between end of re-infusion and beginning of sampling of adrenal blood were only 5 and 9 min. Both dogs were in good condition, and a second haemorrhage of 20 ml./kg was well tolerated and increased aldosterone secretion. In the other two dogs (256, 257) restoration of the initial blood volume did not inhibit secretion of aldosterone, in dog 256 secretion even rose. In these dogs, a longer interval had been allowed between restoration of the blood volume and sampling of adrenal vein blood. This proved to be a highly damaging procedure because the dogs bled heavily from all wounds during the interval. As a result, the second haemorrhage was poorly tolerated and did not constitute blood withdrawal from a starting point of normal blood volume but of already greatly reduced blood volume; damage to the adrenals is suggested by the fall in glucocorticoid secretion during the last collecting period.

These experiments do not support the idea that freshly shed blood contains any glomerulotrophins disappearing on storage and detectable under the conditions of these experiments. The observation that infusion of shed blood, fresh or stale, may fail to lower aldosterone secretion is best explained by the heavy wound bleeding frequently elicited by these infusions, particularly if long intervals are allowed between collection periods. Another possibility will be dealt with at the end of this paper.

Conclusions

When these experiments were carried out information on secretion rates of *ACTH* in stressed dogs was not available, so that the infusion rates had to be chosen arbitrarily. An investigation into the rate at which *ACTH* is secreted by the dog under the conditions of these experiments (Holzbauer 1964) has since shown it to lie between 0·03 and 0·3 m-u. min^{-1} kg^{-1}. Glucocorticoid secretion was stimulated maximally by 0·3 m-u. min^{-1} kg^{-1},

but aldosterone secretion could be further increased by *ACTH* infusions of 3 m-u. min^{-1} kg^{-1}.

In some of the foregoing experiments the amount of hormone infused must have exceeded, in others it will have equalled the endogenous production. The infusions were found to increase aldosterone secretion in four out of 22 dogs; glucocorticoid secretion

TABLE 3. CORTICOSTEROID SECRETION IN DOGS WITH INTACT PITUITARY GLANDS SUBJECTED TO TWO HAEMORRHAGES SEPARATED BY RESTORATION OF THE INITIAL BLOOD VOLUME WITH ONE-DAY-OLD BLOOD

Constant infusion of *ACTH* (0·2 m-u. min^{-1} kg^{-1}) started shortly before the initial haemorrhage. Adrenal blood collected for periods of 20 to 25 min. (Flank incision, aldosterone figures corrected for losses.)

dog no.	body wt. (kg)	adrenal blood sample no.	conditions	*ACTH* infusion	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change) aldosterone	cortisol + corticosterone	adrenal blood flow (ml./h)	mean b.p. (mmHg)	Mayer waves	Ht (% red cells)	plasma conc. (m-equiv./l.) Na^+	K^+	blood withdrawn (ml./kg)
256, male	13·1	S_1	after first haemorrhage	till end of S_2	16·5	1032	307	94	+	59	143	3·3	1st haem: 20
					+78	+52							
		S_2	started 45 min after restoration of blood volume		29·4	1572	194	136	−	49	141	4·0	.
										50	139	4·3	.
					+ 3	−29							
		S_3	after second haemorrhage		30·4	1117	206	73	+	48	138	3·8	2nd haem: 12
257, male	13·4	S_1	after first haemorrhage	till start of S_2	14·6	1449	456	160	−	48	141	3·7	1st haem: 20
					+ 7	+ 1							
		S_2	started 45 min after restoration of blood volume		15·6	1467	327	174	−	52	141	3·5	.
					−37	−20							
		S_3	after second haemorrhage		9·8	1167	201	90	+	53	139	3·7	2nd haem: 16
258, male	12·3	S_1	after first haemorrhage	throughout	10·9	1680	390	90	−	57	137	2·9	1st haem: 20
					−22	−39				55	135	2·9	
		S_2	started 5 min after restoration of blood volume		8·5	1022	276	160	−	54	132	3·0	.
										51	139	3·4	.
					+88	+31							
		S_3	after second haemorrhage		16·0	1339	264	130	−	51	139	3·7	2nd haem: 20
259, male	13·4	S_1	after first haemorrhage	throughout	38·3	1008	173	148	−	52	133	3·6	1st haem: 22
					−27	+ 2							
		S_2	started 9 min after restoration of blood volume		27·8	1032	173	160	−	52	134	3·6	.
										54	137	4·0	.
					+25	−14							
		S_3	after second haemorrhage		34·8	888	144	135	−	54	137	3·5	2nd haem: 21

never rose. The mean secretion rates of both types of corticosteroids in response to infusion of *ACTH* were often found to be lower in the present experiments on non-hypophysectomized dogs than in acutely hypophysectomized dogs; work on this problem will be published separately.

Haemorrhage, superimposed on a constant infusion of *ACTH*, stimulated aldosterone output in 13 out of 28 dogs. These results could have two reasons: either bleeding caused a sudden release of much larger quantities of *ACTH* than expected, or the rise in aldosterone was caused by a factor different from *ACTH*. This conclusion is much more likely and agrees with results on hypophysectomized dogs.

II. Aldosterone secretion after haemorrhage in hypophysectomized dogs maintained with *ACTH*

Ganong & Mulrow (1961), and Davis, Carpenter, Ayers, Holman & Bahn (1961) observed that hypophysectomy does not abolish the increase in aldosterone secretion after haemorrhage. The interpretation of results obtained on hypophysectomized dogs is complicated by the very low secretion rate of all adrenal steroids and the fact that the raised secretion rate obtained after haemorrhage remains far below the pre-haemorrhage figure of the non-hypophysectomized, stressed dog. Experiments were therefore carried out in order to investigate whether increases in aldosterone of the order of magnitude observed in our experiments can be obtained after haemorrhage in the absence of the pituitary gland. For this purpose *ACTH* was infused at a rate of less than one third of that expected to be secreted during operative stress. Infusion at a constant speed was started 10 or 40 min before the adrenal vein was cannulated and continued to the end of the experiment.

Procedures and observations

Shorter periods of ACTH *infusion*

In six dogs *ACTH* infusion (0·01 m-u. min^{-1} kg^{-1}) was started 10 min before the collection of a control sample and continued throughout the experiment. After the first collection period the dogs were bled (9 to 22 ml./kg) and a second sample was collected.

Table 4 shows that haemorrhage increased aldosterone secretion in four out of six dogs by amounts ranging from 52 to 149 %. The two dogs which failed to react had a high initial aldosterone secretion rate, and it is interesting that this was of the same order as that often found in 'non-reactors' with intact pituitaries.

During the control periods, aldosterone and glucocorticoid secretion rate (figure 3, open circles) lay approximately on the extrapolated dose-response curve obtained earlier (Holzbauer 1964) for the relationship between infusion rates of *ACTH* (applied for shorter periods) and secretion of corticoids. After haemorrhage, however, two of the 'reactors' showed considerable increases in glucocorticoid secretion (table 4). This suggested the possibility that the period of *ACTH* infusion before haemorrhage might not have been long enough to obtain a steady adrenal response to *ACTH*.

Longer periods of ACTH *infusion*

In order to ensure that adrenal secretion had reached a steady state before haemorrhage, longer infusion periods were used in a group of six dogs. Before the collection of the initial sample, *ACTH* was infused for 40 min and the speed of the infusion doubled for the first 10 min (0·02 m-u. min^{-1} kg^{-1}); furthermore, two consecutive control samples (S_1 and S_2, table 5) were taken before bleeding. The amount of *ACTH* infused was checked after each collection period.

As shown in table 5 and figure 3 (points marked ●), the glucocorticoid secretion rates during the two control periods fitted the original dose-response curve well and there was no increase during the second samples. The mean aldosterone secretion, however, though equal in the two control samples, was higher than after the brief infusions and corresponded to values obtained previously when *ACTH* was infused for a short time at 3 times the rate.

TABLE 4. EFFECT OF HAEMORRHAGE ON CORTICOSTEROID SECRETION IN HYPOPHYSECTOMIZED DOGS, MAINTAINED ON A CONTINUOUS i.v. INFUSION OF 0·01 m-u. *ACTH* min^{-1} (kg body wt)$^{-1}$

Hypophysectomy 2 h, start of *ACTH* infusion 10 min before collection of first adrenal blood sample (S_1); second sample (S_2) after haemorrhage. (Flank incision, adrenal blood collected for periods of 30 min, aldosterone figures corrected for losses.)

dog no.	body wt. (kg)	adrenal blood sample no.	adrenocortical secretion rates adrenal (μg (g adrenal)$^{-1}$ h^{-1} and % change) aldosterone	cortisol + corticosterone	adrenal blood flow (ml./h and % change)	mean b.p. (mmHg)	Mayer waves	Ht (% red cells)	blood withdrawn before S_2 (ml./kg)	100 m-equiv. Na^+/day for (no. of days)
				Group I. Reactors						
345, female	8·5	S_1	6·7	1069	186	110	–	47	14	16
		S_2	16·7 + 149	1222 + 14	180 − 3	70	–	49		.
347, female	6·8	S_1	5·3	294	114	140	–	48	22	17
		S_2	12·2 + 129	569 + 94	144 + 26	100	+	51		.
350, female	7·0	S_1	6·9	147	160	85	–	47	13	24
		S_2	10·5 + 52	141 − 4	98 − 40	58	–	52		.
351, male	12·4	S_1	8·7	1092	444	135	–	56	22	3
		S_2	14·1 + 62	1643 + 50	314 − 29	120	+	58		.
mean ± s.e.		S_1	6·9 ± 0·7	651 ± 250	226 ± 74	118 ± 13	.	.	17·8 ± 2·5	.
		S_2	13·4 ± 1·3	894 ± 334	184 ± 46	87 ± 14	.	.		.
				Group II. Non-reactors						
346, male	9·8	S_1	14·7	1132	389	135	–	51	16	17
		S_2	14·4 − 2	1024 − 9	206 − 47	75	–	47		.
348, female	10·3	S_1	9·7	1027	288	105	–	58	9	16
		S_2	12·1 + 25	1170 + 14	224 − 22	70	–	53		.
mean		S_1	12·2	1080	339	120	.	.	12·5	.
		S_2	13·3	1097	215	73	.	.		.
combined mean ± s.e. of all dogs		S_1	8·7 ± 1·4	794 ± 183	264 ± 54	118 ± 8·9	.	51 ± 2·0	16 ± 2·1	.
		S_2	13·3 ± 0·9	962 ± 217	194 ± 30	82 ± 9·5	.	52 ± 1·5		.

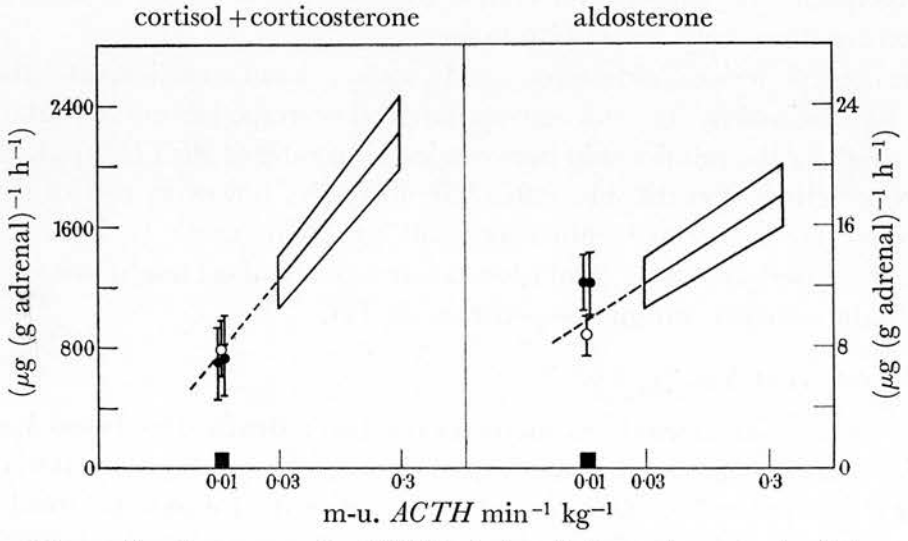

FIGURE 3. Steroid secretion in response to *ACTH* infusions in hypophysectomized dogs. (Same batch of *ACTH* used on all occasions.) Enclosed areas: mean responses (± s.e.) obtained in a previous set of experiments (Holzbauer 1964) when *ACTH* was infused for 20 to 30 min, starting 5 min before the start and ending 5 min before the end of each collection period. ○, ●, Present experiments: *ACTH* infusion started 10 (○) or 40 (●) min before the first blood collection and continued throughout experiment; during the first 10 min of the 40 min infusion period *ACTH* was administered at twice the speed of that used subsequently. The paired black dots correspond to samples collected consecutively (S_1 and S_2 of table 5). All samples were taken before bleeding.

In spite of the high initial values, haemorrhage increased aldosterone secretion further in five of six dogs (table 5). The two largest increases (64 and 174 %, experiments 354 and 357) were, as in the previous group, associated with large rises in cortisol or glucocorticoid secretion.

TABLE 5. EFFECT OF HAEMORRHAGE ON CORTICOSTEROID SECRETION IN HYPOPHYSECTOMIZED DOGS MAINTAINED ON A CONTINUOUS INFUSION OF 0·01 m-u. *ACTH* min^{-1} kg^{-1} (0·02 FOR THE FIRST 10 min)

Hypophysectomy 2 h, start of *ACTH* infusion 40 min before collection of first adrenal blood sample (S_1); second sample (S_2) immediately following S_1, third sample (S_3) after haemorrhage. (Flank incision, adrenal blood collected for periods of 30 min, aldosterone figures corrected for losses.)

dog no.	body wt. (kg)	adrenal blood sample no.	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change): aldosterone	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change): cortisol + corticosterone	adrenal blood flow (ml./h and % change)	mean b.p. (mmHg)	Mayer waves	Ht (% red cells)	blood withdrawn before S_3 (ml./kg)	100 m-equiv. Na^+/day for (no. of days)
Group I. Reactors										
354, male	13·4	S_1	12·3	506*	172	165	−	.	.	55
		S_2	14·7 + 20	114 − 78*	192	170	−	.	.	.
		S_3	24·1 + 64	931 +717	146 −24	80	+	.	19	.
355, male	10·5	S_1	8·0	921*	154	155	−	45	.	3
		S_2	7·3 − 9	927 0*	174	145	−	48	.	.
		S_3	10·1 + 38	1149 + 24	144 −17	80	+	45	15	.
356, female	9·3	S_1	20·4	594*	250	135	+	55	.	3
		S_2	20·6 + 1	325 − 45*	240	105	+	50	.	.
		S_3	31·1 + 51	364 + 12	170 −29	90	+	48	23	.
357, male	10·1	S_1	8·6	294	222	120	−	46	.	8
		S_2	8·0 − 7	190 − 35	288	115	−	47	.	.
		S_3	21·9 +174	834 +339	146 −49	80	−	46	19	.
359, female	11·4	S_1	10·1	1147	184	120	−	31	.	10
		S_2	8·8 − 13	1083 − 6	150	110	+	51	.	.
		S_3	13·7 + 56	724 − 33	128 −15	70	−	52	11	.
mean ± S.E.		S_1	11·9 ± 2·3	721	196 ± 17	139 ± 9	.	.	.	.
		S_2	11·9 ± 2·5	637	209 ± 25	129 ± 12	.	.	.	.
		S_3	20·2 ± 3·7	779	147 ± 7	80 ± 3	.	.	17·4 ± 2	.
Group II. Non-reactor										
358, female	10·4	S_1	13·5	629	118	130	−	38	.	9
		S_2	13·5 0	928 + 48	188	115	−	43	.	.
		S_3	16·9 + 25	1793 + 93	110 −41	85	−	42	19	.
combined mean ± S.E. of all dogs		S_1	12·2 ± 1·9	690 ± 248	183 ± 19	138 ± 7·6	.	.	.	.
		S_2	12·2 ± 2·1	734 ± 275	205 ± 21	127 ± 10·4	.	.	.	.
		S_3	19·6 ± 3·1	1117 ± 339	141 ± 8	81 ± 2·7	.	.	18 ± 1·7	.

* Cortisol only; results not included in the mean.

Though the circulatory conditions were somewhat better in the second group of dogs, in both groups the mean arterial pressure was lower than in intact dogs. Mayer waves were less frequent after haemorrhage or of smaller amplitude. Haematocrits and plasma electrolytes showed the same small changes in the course of the experiment which have been reported for dogs with intact pituitary. One reactor (354) and the non-reactor (358) showed signs of disease: the first dog had tape worms and diarrhoea, the second was anaemic and its blood sedimentation rate was high.

Conclusions

Out of 12 hypophysectomized dogs, in which adrenal steroid synthesis was maintained at a submaximal rate by infusions of *ACTH*, nine responded to haemorrhage by increased aldosterone output. Initial levels and increments in aldosterone secreted were similar to those observed in dogs with intact pituitaries, but the number of reactors, 75 % instead of 50 %, was greater after hypophysectomy. This is probably a consequence of being able, after hypophysectomy, to control the *ACTH* available to the adrenal gland and thus keep initial aldosterone secretion sufficiently low to allow an increase after haemorrhage. The large increase in cortisol secretion observed in four dogs after haemorrhage cannot be accounted for by an increase in blood *ACTH* concentration due to a reduction in blood volume; this would amount to 25 % at most, and changes in *ACTH* concentration of 25 % would not lead to measurable changes in steroid output (see figure 3). Since in these experiments basal glucocorticoid secretion was kept lower than the value found in stressed dogs with intact pituitaries, it is conceivable that the action on glucocorticoids of an aldosterone releasing factor became noticeable, while it previously escaped detection.

Whereas the results confirm the view that release of *ACTH* is not essential for the occurrence of a rise in aldosterone secretion after bleeding, there is one other factor which could account for both the larger percentage of reactors in the hypophysectomized dog and the occasional rise in glucocorticoids after bleeding. It is feasible that haemorrhage interferes with the distribution and fate of the infused *ACTH*. An effect of haemorrhage on the fate or action of an injected substance was observed by Scornik & Paladini (1964). These authors injected equal doses of renin into nephrectomized dogs before and after bleeding and measured the blood angiotensin concentration. It was found to be much higher after haemorrhage than before.

III. Aldosterone secretion before and after haemorrhage in nephrectomized dogs

The investigations of Davis and his colleagues have provided evidence for a part played by the renin-angiotensin system in the initiation of high aldosterone secretion rates in hypophysectomized dogs suffering from malignant hypertension (Davis, Carpenter & Ayers 1962) or from chronic heart failure caused either by constriction of the inferior vena cava (Davis, Ayers & Carpenter 1961) or by a large arteriovenous shunt (Davis, Urquhart, Higgins, Rubin & Hartroft 1964). Rises in aldosterone secretion after caval constriction were also seen in hypophysectomized dogs with kidneys deprived of their nerve supply by transplantation (Carpenter, Davis, Holman, Ayers & Bahn 1961). Furthermore, a rise in the secretion rates of aldosterone and also of glucocorticoids was produced by infusions of angiotensin II in dogs (Mulrow & Ganong 1961; Carpenter, Davis & Ayers 1961; Bartter, Casper, Delea & Slater, 1961), in man (Biron, Koiw, Nowaczynski, Brouillet & Genest 1961; Laragh, Angers, Kelly & Lieberman 1960), and in the sheep (Blair-West, Coghlan, Denton, Goding, Munro, Peterson & Wintour 1962).

It is uncertain whether angiotensin exerts a significant control over the adrenal cortex under more physiological conditions. In dogs, the rise in aldosterone secretion caused by sodium depletion seems to depend on the presence of the kidneys (Davis, Ayers &

Carpenter 1961), but the pituitary is also involved: the increase in aldosterone secretion during sodium depletion in conscious dogs is smaller when the pituitary gland is absent (Binnion, Davis, Brown & Olichney 1965). In the sheep the kidney does not seem to be indispensable for the response to sodium loss (Blair-West, Coghlan, Denton, Goding, Wintour & Wright 1963), and the same appears to hold for the rat (Cade & Perenich 1965). The effect of potassium on aldosterone secretion is independent of the kidneys in both species (Davis, Urquhart & Higgins 1963; Blair-West *et al.* 1963).

From experiments on hypophysectomized-nephrectomized dogs, it has also been concluded that the kidneys mediate the rise in aldosterone secretion evoked by haemorrhage (Ganong & Mulrow 1961, 1962; Davis, Carpenter, Ayers, Holman & Bahn 1961). This possibility is supported by the fact that haemorrhage releases renin from the kidneys (Huidobro & Braun-Menendez 1942; Scornik & Paladini 1964; Regoli & Vane 1964).

In the following experiments the effect of haemorrhage on the adrenal cortex was studied in the absence of the kidneys but in the presence of the pituitary gland.

Procedures and observations

Effect of acute bilateral nephrectomy

The object of the first experiments was to see whether nephrectomy would affect basal aldosterone secretion. A control sample of adrenal blood was taken in four dogs. Both kidneys were then removed and the collection of a second sample was started 37 to 44 min, that of a third sample 55 to 94 min later. Forty minutes after nephrectomy, rises in aldosterone secretion of 55 and 48 % were observed in two dogs; these were not maintained; no changes occurred in the remaining two dogs. In another two experiments adrenal blood was collected only after nephrectomy, four consecutive samples being taken in one and two in the other dog. There was no change in aldosterone secretion when up to four consecutive samples were examined between 0·5 and 3 h after nephrectomy. Glucocorticoid secretion remained constant. Rises in the plasma potassium concentration in the course of the experiments were of the same order of magnitude as those observed in non-nephrectomized dogs. The two increases in aldosterone secretion seen after nephrectomy were probably a consequence of the surgical trauma and due to increased release of *ACTH* and renin; when the immediate effect of the surgery had passed, aldosterone secretion remained steady in the nephrectomized dog.

Dogs bled once

In seven experiments control samples were collected before and after bilateral nephrectomy. The dogs were then bled ($22 \pm 1{\cdot}0$ ml./kg) and a post-haemorrhage sample was taken. The results are shown in table 6. In six dogs aldosterone secretion was slightly elevated 26 to 38 min after nephrectomy, but three dogs responded to haemorrhage with a further rise in aldosterone secretion of 31, 40 and 149 %. In the four dogs which failed to respond, the mean aldosterone secretion before bleeding was higher than in the reactors. Circulatory conditions remained satisfactory after haemorrhage. Glucocorticoid secretion rates and blood pressure remained unchanged in all dogs, but adrenal blood flow decreased. There were no abnormalities in the plasma concentrations of Na^+ and K^+.

In another group of dogs the experiments were shortened by removing both kidneys at

the beginning of the experiment and collecting only one control sample 26 to 88 min later. Then the dogs were bled and a second sample taken. The results are given in tables 7 and 8. In table 8 aldosterone figures are corrected for losses.

TABLE 6. THE EFFECT OF ACUTE BILATERAL NEPHRECTOMY ON CORTICOSTEROID SECRETION AND ON THE RESPONSE OF ALDOSTERONE SECRETION TO HAEMORRHAGE

First adrenal blood sample (S_1) collected 30 min after end of adrenal vein cannulation, second sample (S_2) started 26 to 38 min after nephrectomy, third sample (S_3) after bleeding. Arterial blood samples: A_1 at start of S_1, A_2 at end of haemorrhage, A_3 at end of S_3. (Aldosterone figures not corrected for losses.)

dog no.	body wt. (kg)	adrenal blood sample no.	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change): aldosterone	cortisol + corticosterone	adrenal blood flow (ml./h)	mean b.p. (mmHg)	Mayer waves	arterial blood samples	Ht (% red cells)	plasma conc. (m-equiv./l.) Na^+	plasma conc. (m-equiv./l.) K^+	blood withdrawn before S_3 (ml./kg)	Na^+ intake/day: m-equiv.	Na^+ intake/day: no. of days
Group I. Reactors														
172, male	12·3	S_1	7·9	1793	158	166	−	A_1	59	141	3·5	.	170	12
		S_2	10·2 + 29	1811 + 1	132	170	−	A_2	58	136	5·0	.	100	11
		S_3	13·4 + 31	1819 0	96	125	−	A_3	56	136	7·0	23	.	.
174, male	12·6	S_1	4·3	1761	180	136	−	A_1	49	138	3·7	.	170	5
		S_2	4·5 + 5	1808 + 3	144	142	−	A_2	49	138	4·8	.	100	25
		S_3	11·2 + 149	1675 − 7	55	90	+	A_3	55	138	5·2	20	.	.
175, female	10·8	S_1	6·9	1389	202	126	−	A_1	50	138	3·9	.	170	5
		S_2	8·0 + 16	1295 − 7	96	138	−	A_2	41	134	5·3	.	100	31
		S_3	11·2 + 40	1234 − 5	125	115	+	A_3	43	136	5·4	25	.	.
mean ± S.E.		S_1	6·4 ± 1·1	1648 ± 130	180 ± 13	143 ± 12	.	A_1	53 ± 3·2	139 ± 1·0	3·7 ± 0·1	.	.	.
		S_2	7·6 ± 1·7	1638 ± 172	124 ± 14	150 ± 10	.	A_2	49 ± 4·9	136 ± 1·1	5·0 ± 0·1	.	.	.
		S_3	11·9 ± 0·7	1576 ± 176	92 ± 20	110 ± 10	.	A_3	51 ± 4·2	137 ± 0·7	5·9 ± 0·6	23 ± 1	.	.
Group II. Non-reactors														
180, female	15·4	S_1	8·4	1968	305	150	−	A_1	68	145	3·3	.	100	10
		S_2	9·6 + 14	1860 − 5	298	150	−	A_2	66	153	4·2	.	.	.
		S_3	10·6 + 10	1776 − 5	293	110	+	A_3	61	148	4·5	26	.	.
183, male	15·0	S_1	8·6	2465	269	162	−	A_1	53	139	4·4	.	100	10
		S_2	9·7 + 13	2361 − 4	228	168	−	A_2	51	136	5·6	.	.	.
		S_3	10·2 + 5	2312 − 2	174	95	+	A_3	52	147	5·3	20	.	.
186, female	17·2	S_1	10·5	1875	168	138	−	A_1	56	145	3·5	.	100	1
		S_2	10·5 0	1942 + 4	108	152	−	A_2	53	142	5·4	.	.	.
		S_3	10·6 + 1	1868 − 4	91	120	−	A_3	51	145	5·8	23	.	.
189, male	12·0	S_1	6·4	1611	240	148	−	A_1	60	144	3·4	.	100	4
		S_2	7·0 + 9	1456 − 10	112	145	−	A_2	65	142	4·7	.	.	.
		S_3	8·1 + 16	. .	168	100	+	A_3	62	147	4·0	20	.	.
mean ± S.E.		S_1	8·5 ± 0·8	1980 ± 179	246 ± 29	150 ± 4·8	.	A_1	59 ± 3·3	143 ± 1·4	3·7 ± 0·3	.	.	.
		S_2	9·2 ± 0·8	1905 ± 186	187 ± 46	154 ± 5·0	.	A_2	59 ± 3·9	143 ± 3·5	5·0 ± 0·3	.	.	.
		S_3	9·9 ± 0·6	1985 ± 165	182 ± 42	106 ± 5·5	.	A_3	57 ± 2·9	147 ± 0·6	4·9 ± 0·4	22 ± 1·4	.	.
groups I and II combined mean ± S.E.		S_1	7·6 ± 0·7	1837 ± 127	217 ± 21	147 ± 5·0	.	A_1	56 ± 2·5	141 ± 1·2	3·7 ± 0·1	.	.	.
		S_2	8·5 ± 0·8	1813 ± 122	160 ± 28	152 ± 4·6	.	A_2	55 ± 3·4	140 ± 2·4	5·0 ± 0·2	.	.	.
		S_3	10·8 ± 0·6	1781 ± 142	143 ± 30	108 ± 5·0	.	A_3	54 ± 2·4	142 ± 2·1	5·3 ± 0·3	22 ± 1·0	.	.

Out of 11 nephrectomized dogs shown in table 7, five responded to haemorrhage by an increase in aldosterone secretion ranging from 31 to 62 %, whereas there was no change in the glucocorticoids. Dog 139 produced the rise in aldosterone in spite of a low blood pressure, a haematocrit of 75 % and a high initial secretion rate. However, in this animal plasma potassium increased by 2·7 m-equiv./l. in the course of the experiment. This increase was unusually large and may have contributed to the rise in aldosterone secretion.

TABLE 7. EFFECT OF ACUTE BILATERAL NEPHRECTOMY ON THE RESPONSE OF ALDOSTERONE SECRETION TO HAEMORRHAGE

Collection of first adrenal blood sample (S_1) started 26 to 84 min after nephrectomy, second sample (S_2) after bleeding. Arterial blood samples for haematocrit and plasma K^+ and Na^+ estimation taken at beginning of S_1 (A_1) and end of S_2 (A_2). (Aldosterone figures not corrected for losses.)

dog no.	body wt. (kg)	adrenal blood sample no.	time between the removal of the 2nd kidney and S_1 (min)	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change) aldosterone	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change) cortisol + corticosterone	adrenal blood flow (ml./h)	mean b.p. (mmHg)	Mayer waves	arterial blood samples	Ht (% red cells)	plasma conc. (m-equiv./l.) Na^+	plasma conc. (m-equiv./l.) K^+	blood withdrawn before S_2 (ml./kg)	Na^+ intake/day m-equiv.	Na^+ intake/day no. of days
Group I. Reactors															
134, male	8·8	S_1	32	2·1	1879	290	114	−	A_1	.	.	.	.	30	9
		S_2	.	3·4 +62	1384 −26	129	70	+	A_2	.	.	.	20	.	.
139, male	7·8	S_1	28	10·6	1367	154	77	−	A_1	75	160	3·8	.	30	1
		S_2	.	15·3 +42	1233 −10	90	48	+	A_2	67	168	6·5	21	.	.
194, male	8·9	S_1	84	3·0	1284	233	155	−	A_1	61	148	3·9	.	100	16
		S_2	.	4·8 +60	1300 + 1	154	98	+	A_2	59	157	4·1	19	.	.
195, female	11·0	S_1	67	3·7	1195	136	140	−	A_1	58	157	3·0	.	100	16
		S_2	.	5·5 +49	1328 +11	92	120	+	A_2	51	152	4·9	21	.	.
197, female	18·3	S_1	70	6·4	1565	230	166	+	A_1	58	157	3·2	.	100	3
		S_2	.	8·4 +31	1565 0	168	80	+	A_2	51	153	3·4	13	.	.
mean ±S.E.		S_1	.	5·2 ± 1·5	1458 ± 122	209 ± 28	130 ± 16	.	A_1	63 ± 4·1	156 ± 2·6	3·5 ± 0·2	.	.	.
		S_2	.	7·5 ± 2·1	1362 ± 56	127 ± 16	83 ± 12	.	A_2	57 ± 3·8	158 ± 3·7	4·7 ± 0·7	19 ± 1·5	.	.
Group II. Non-reactors															
137, male	8·5	S_1	37	3·2	1636	213	100	+	A_1	52	149	2·9	.	30	2
		S_2	.	2·8 −13	1529 − 7	144	65	−	A_2	49	139	4·0	19	.	.
138, male	13·7	S_1	26	8·7	1415	169	153	+	A_1	56	165	4·0	.	30	1
		S_2	.	10·4 +20	1213 −14	84	65	−	A_2	56	150	6·1	11	.	.
153, male	11·9	S_1	30	10·5	1515	214	133	+	A_1	60	150	3·9	.	30	42
		S_2	.	11·9 +13	1518 0	108	65	−	A_2	58	144	5·1	17	.	.
190, male	12·3	S_1	75	7·4	2073	254	118	−	A_1	62	150	3·4	.	100	16
		S_2	.	8·3 +12	2245 + 8	152	64	+	A_2	57	147	3·7	17	.	.
191, female	9·9	S_1	61	7·4	1483	180	155	−	A_1	55	150	4·7	.	100	16
		S_2	.	9·6 +30	1745 +18	199	95	+	A_2	49	143	5·3	20	.	.
193, female	15·5	S_1	80	10·7	1898	274	132	−	A_1	55	143	4·8	.	100	10
		S_2	.	10·2 − 5	2078 + 9	240	90	+	A_2	52	138	5·9	16	.	.
mean ±S.E.		S_1	.	7·6 ± 1·0	1673 ± 90	215 ± 14	130 ± 7·4	.	A_1	57 ± 1·1	152 ± 3·0	4·1 ± 0·2	.	.	.
		S_2	.	8·5 ± 1·2	1694 ± 135	153 ± 20	72 ± 5·5	.	A_2	53 ± 1·4	145 ± 3·0	5·0 ± 0·3	16 ± 1·1	.	.
groups I and II combined. mean ±S.E.		S_1	.	6·6 ± 0·9	1584 ± 76	213 ± 14	130 ± 7·5	.	A_1	59 ± 1·8	154 ± 2·0	3·9 ± 0·2	.	.	.
		S_2	.	8·1 ± 1·1	1556 ± 94	142 ± 13	77 ± 6·0	.	A_2	55 ± 1·6	149 ± 2·9	4·9 ± 0·3	17 ± 1·0	.	.

In four of the six non-reactors circulation was unsatisfactory. The fifth, 191, which showed a rise of 30 %, was pregnant; all these dogs except 137 had a high initial aldosterone secretion rate.

The four dogs listed in table 8 follow the same pattern, two showing increases in aldosterone secretion after haemorrhage, and two failing to respond; one of the non-reactors (dog 261) had poorly arterialized blood and a high initial aldosterone secretion; the poor response of dog 262 had no obvious reason.

TABLE 8. EFFECT OF ACUTE BILATERAL NEPHRECTOMY ON THE RESPONSE OF ALDOSTERONE SECRETION TO HAEMORRHAGE

Collection of first adrenal blood sample (S_1) started 70 to 88 min after nephrectomy, second sample (S_2) after haemorrhage. Arterial blood samples for haematocrit and plasma K^+ and Na^+ estimation taken at the beginning of S_1 and at the end of S_2. (Aldosterone figures corrected for losses.)

dog no.	body wt. (kg)	adrenal blood sample no.	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change): aldosterone	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change): cortisol+corticosterone	adrenal blood flow (ml./h)	mean b.p. (mmHg)	Mayer waves	Ht (% red cells)	plasma conc. (m-equiv./l.): Na^+	plasma conc. (m-equiv./l.): K^+	blood withdrawn before S_2 (ml./kg)	100 m-equiv. Na^+/day for (no. of days)
Group I. Reactors												
263, male	10·8	S_1	13·3	1252	228	153	(+)	65	138	3·2	.	24
		S_2	19·9 +50	1367 + 9	132	96	+	55	143	4·6	20	.
264, male	17·0	S_1	13·8	1192	360	122	+	67	145	2·6	.	24
		S_2	19·8 +43	1184 − 1	139	70	+	63	145	3·2	19	.
Group II. Non-reactors												
261, male	11·0	S_1	18·9	1232	252	146	+	58	138	4·5	.	26
		S_2	20·1 + 6	1008 −18	202	90	+	59	145	4·1	20	.
262, male	14·2	S_1	10·2	1253	426	164	−	65	148	3·2	.	26
		S_2	13·1 +28	1175 − 6	270	118	+	.	.	.	23	.
mean ± S.E.		S_1	14·1 ± 1·8	1232 ± 14	317 ± 46	146 ± 8·9	A_1	64 ± 2·0	142 ± 2·5	3·4 ± 0·4	.	.
		S_2	18·2 ± 1·7	1184 ± 73	186 ± 32	94 ± 9·9	A_2	59 ± 2·3	144 ± 0·7	4·0 ± 0·4	21 ± 0·9	.

Dogs bled twice

The difficulty experienced in using a response which, even with the kidneys present, may only be seen in 50 % of the animals, prompted attempts at bleeding the same dog twice, once before and once after nephrectomy.

In three dogs (table 9) the kidneys were mobilized from their surroundings and ligatures placed loosely underneath the main vessels. After collection of a control sample of adrenal blood, the dogs were bled and a second sample was taken. Then the shed blood was re-infused, and after an interval of 45 to 60 min both kidneys were removed; 30 to 36 min later a third blood sample was collected, the dogs were bled again and a final sample was taken. In the first experiment (dog 162), re-infusion of blood followed by nephrectomy reduced aldosterone secretion below the initial level, and the second haemorrhage produced a significant rise, output of glucocorticoids remaining steady throughout. In the next dog (166), circulatory conditions did not remain satisfactory to the end, and the amount of blood loss tolerated after nephrectomy was less than half the previous amount. No significant increase in aldosterone production was found after the second haemorrhage. The third dog (171) bled heavily throughout the experiment, and did not tolerate withdrawal of the

TABLE 9. COMPARISON OF THE EFFECT OF HAEMORRHAGE ON ALDOSTERONE SECRETION IN THE SAME DOG BEFORE AND AFTER BILATERAL NEPHRECTOMY

First arterial blood sample (A_1) taken at beginning of S_1, second (A_2) after first haemorrhage, third (A_3) after second haemorrhage, fourth (A_4) at the end of S_4. (Aldosterone figures not corrected for losses.)

dog no.	body wt. (kg)	adrenal blood sample no.	conditions	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change) aldosterone	cortisol + corticosterone	adrenal blood flow (ml./h)	mean b.p. (mmHg)	Mayer waves	arterial blood samples	Ht (% red cells)	plasma conc. (m-equiv./l.) Na^+	K^+	blood withdrawn (ml./kg)	100 m-equiv. Na^+/day for (no. of days)
162, male	7·5	S_1	control	7·2	1368	140	108	−	A_1	52	140	3·9	.	8
		S_2	after 1st haemorrhage	9·2 +28	1695 +24	98	75	+	A_2	50	141	4·1	1st: 13	.
		S_3	80 min after re-infusion of blood, 30 min after bilateral nephrectomy	5·8 −37 / +48	1376 −19 / + 4	127	110	−	A_3	55	141	4·7	.	.
		S_4	after 2nd haemorrhage	8·6	1428	90	86	+	A_4	52	140	5·2	2nd: 15	.
166, male	15·1	S_1	control	3·7	1554	315	145	−	A_1	64	147	3·6	.	5
		S_2	after 1st haemorrhage	6·1 +65	1454 − 6	234	80	+	A_2	51	146	5·3	1st: 19	.
		S_3	75 min after re-infusion of blood, 30 min after bilateral nephrectomy	4·6 −25 / +13	1438 − 1 / − 8	282	124	−	A_3	58	146	5·2	.	.
		S_4	after 2nd haemorrhage	5·2	1323	126	60	−	A_4	55	145	5·6	2nd: 9	.
171, female	16·4	S_1	control	2·9	996	300	155	+	A_1	58	136	4·5	.	10
		S_2	after 1st haemorrhage	2·3 −21	924 − 7	180	67	+	A_2	56	137	5·0	1st: 11	.
		S_3	96 min after re-infusion of blood, 36 min after bilateral nephrectomy	3·0 +30 / −40	912 − 2 / −20	237	134	+	A_3	49	141	4·9	.	.
		S_4	after 2nd haemorrhage	1·8	732	99	48	−	A_4	46	139	4·4	2nd: 8	.
mean ±S.E.		S_1		4·6 ± 1·3	1306 ± 164	252 ± 56	136 ± 14·0	.	A_1	58 ± 3·5	141 ± 3·2	4·0 ± 2·6	1st:	.
		S_2		5·9 ± 2·0	1358 ± 228	171 ± 40	74 ± 3·7	.	A_2	52 ± 1·9	141 ± 2·6	4·8 ± 3·6	14 ± 2·4	
		S_3		4·5 ± 0·8	1242 ± 166	215 ± 46	123 ± 7·0	.	A_3	54 ± 2·6	143 ± 1·6	4·9 ± 1·5	2nd:	.
		S_4		5·2 ± 2·0	1161 ± 217	105 ± 11	65 ± 11·0	.	A_4	51 ± 2·6	141 ± 1·9	5·1 ± 3·5	11 ± 2·2	

usual amount of blood even at the first bleeding period; there was a fall in aldosterone secretion after both haemorrhages.

Another modification of the same experiment, in which the right kidney was extirpated before taking the first control sample and a shorter interval allowed after restoration of the initial blood volume, was no more successful in producing a sufficiently low secretion rate of aldosterone before the second haemorrhage and creating conditions which would have allowed a significant rise afterwards. It was therefore decided to try and reduce the operative stress by removing the first kidney at a preliminary aseptic operation. This not only shortened the operation, but avoided the stress of a midline incision.

Effect of extirpation of left kidney after preliminary right nephrectomy

The right kidney was removed in an aseptic operation under pentobarbitone sodium anaesthesia 2 to 3 weeks before the experiment, at which left nephrectomy and adrenal cannulation were performed through a flank incision. In four dogs blood collection was started 1 h after removal of the left kidney. It can be seen (table 10) that in these animals the circulatory conditions remained satisfactory, but in two dogs (303, 304) initial aldosterone secretion was very high. After bleeding, no rise in aldosterone secretion was observed. The reason might have been that not enough time had been allowed for renin, released during nephrectomy, to disappear from the circulation.

TABLE 10. EFFECT OF HAEMORRHAGE ON ALDOSTERONE SECRETION IN NEPHRECTOMIZED DOGS

Right kidney removed 13 to 15 days before the experiment, left kidney 1 h before the start of the collection of the first adrenal blood sample (S_1); second sample (S_2) after haemorrhage. Arterial blood samples taken at the start of S_1 (A_1) and at the end of S_2 (A_2). (Aldosterone figures corrected for losses.)

dog no.	body wt. (kg)	adrenal blood sample no.	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change): aldosterone	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change): cortisol + corticosterone	adrenal blood flow (ml./h)	mean b.p. (mmHg)	Mayer waves	arterial blood samples	Ht (% red cells)	plasma conc. (m-equiv./l.): Na^+	plasma conc. (m-equiv./l.): K^+	blood withdrawn before S_2 (ml./kg)	100 m-equiv. Na^+/day for (no. of days)
301, male	10·5	S_1	11·3	1576	504	130	–	A_1	51	156	2·4	.	21
		S_2	8·9 − 21	1766 + 12	327	80	–	A_2	48	162	3·3	19	.
302, male	10·0	S_1	12·6	1596	264	165	–	A_1	50	152	3·0	.	15
		S_2	13·0 + 3	1636 + 3	168	110	–	A_2	44	161	4·0	23	.
303, male	24·8	S_1	17·5	1387	692	160	+	A_1	58	156	3·1	.	21
		S_2	15·1 − 14	952 − 31	495	135	+	A_2	53	156	3·3	25	.
304, female	11·9	S_1	22·2	2778	366	182	–	A_1	46	185	3·6	.	38
		S_2	23·8 + 7	3097 + 12	117	95	+	A_2	39	163	3·6	16	.
mean ± S.E.		S_1	15·9 ± 2·5	1834 ± 318	457 ± 93	159 ± 11	.	A_1	51 ± 2·5	162 ± 7·6	3·0 ± 0·3	.	.
		S_2	15·2 ± 3·1	1863 ± 448	277 ± 85	105 ± 12	.	A_2	46 ± 3·0	161 ± 1·6	3·6 ± 0·2	21 ± 2·0	.

Thus, in the remaining 13 dogs an interval of 3 h was allowed between removal of the second kidney and collection of the control sample. As can be seen from table 11, this procedure reduced the initial aldosterone secretion in most instances, and now six dogs responded to haemorrhage with rises ranging from 32 to 133 %. Glucocorticoid secretion was hardly affected. A control group of 10 non-nephrectomized dogs was run at the same time. In these animals a small piece of intestine was removed 3 h before the first collection of adrenal blood to simulate the stress caused by the manipulation during left nephrectomy.

A positive response to haemorrhage was observed in five dogs (for details see part I, table 3). The percentage of responding dogs was, thus, the same in control and in nephrectomized animals.

TABLE 11. EFFECT OF HAEMORRHAGE ON ALDOSTERONE SECRETION IN NEPHRECTOMIZED DOGS

Right kidney removed 21 to 30 days before the experiment, left kidney 3 h before start of collection of the first adrenal blood sample (S_1), second sample (S_2) collected after haemorrhage. Haematocrit and plasma electrolyte concentrations estimated before S_1 (A_1) and at the end of S_2 (A_2). (Aldosterone figures corrected for losses.)

dog no.	body wt. (kg)	adrenal blood sample no.	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change): aldosterone	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change): cortisol + corticosterone	adrenal blood flow (ml./h)	mean b.p. (mmHg)	Mayer waves	arterial blood samples	Ht (% red cells)	plasma conc. (m-equiv./l.): Na^+	plasma conc. (m-equiv./l.): K^+	blood withdrawn before S_2 (ml./kg)	100 m-equiv. Na^+/day for (no. of days)
Group I. Reactors													
310, male	12·4	S_1	9·5	1804	240	140	−	A_1	49	169	4·2	.	31
		S_2	18·5 + 95	2319 + 29	240	90	+	A_2	51	176	4·3	27	.
311, male	12·8	S_1	6·5	1766	139	170	−	A_1	56	171	3·3	.	32
		S_2	9·8 + 51	1738 − 2	89	100	+	A_2	53	162	4·0	20	.
316, female	8·7	S_1	7·6	1592	144	160	−	A_1	47	144	4·1	.	61
		S_2	10·0 + 32	963 − 40	67	120	+	A_2	45	146	5·6	23	.
317, female	9·8	S_1	4·8	1281	434	170	−	A_1	46	149	4·1	.	32
		S_2	7·9 + 65	1641 + 28	240	90	−	A_2	46	144	5·0	20	.
318, male	8·7	S_1	6·5	1375	132	155	−	A_1	50	150	4·7	.	32
		S_2	11·3 + 74	859 − 38	122	90	+	A_2	54	152	4·9	24	.
319, female	14·5	S_1	5·7	2162	323	180	−	A_1	56	146	4·8	.	34
		S_2	13·3 + 133	2229 + 3	216	100	+	A_2	53	152	5·0	21	.
mean ± S.E.		S_1	6·8 ± 0·7	1663 ± 131	235 ± 50	163 ± 5·7	.	.	51 ± 1·7	155 ± 4·9	4·2 ± 0·2	.	.
		S_2	11·8 ± 1·5	1625 ± 251	162 ± 32	98 ± 4·8	.	.	50 ± 1·6	155 ± 4·9	4·8 ± 0·2	23 ± 1·1	.
Group II. Non-reactors													
307, male	14·1	S_1	20·3	2239	259	175	−	A_1	55	175	4·5	.	49
		S_2	16·8 − 17	1950 − 13	209	115	+	A_2	52	162	5·1	22	.
308, female	15·7	S_1	11·0	2070	330	180	−	A_1	65	169	3·8	.	29
		S_2	11·1 + 1	2422 + 17	330	150	+	A_2	63	174	4·4	24	.
313, male	17·0	S_1	8·0	2028	264	155	−	A_1	49	151	4·6	.	64
		S_2	10·4 + 30	1428 − 30	249	100	+	A_2	55	143	4·7	21	.
314, male	12·8	S_1	6·1	1411	264	170	−	A_1	62	143	3·3	.	29
		S_2	5·0 − 18	1190 − 16	147	120	−	A_2	50	145	3·7	27	.
315, female	11·5	S_1	6·5	1171	462	170	−	A_1	58	150	3·3	.	29
		S_2	6·4 − 2	1206 + 3	351	125	−	A_2	48	145	3·7	22	.
320, male	8·1	S_1	2·7	1668	163	195	−	A_1	61	151	4·2	.	24
		S_2	3·4 + 26	1125 − 33	51	100	+	A_2	58	163	5·2	16	.
321, male	17·0	S_1	8·5	1542	257	165	−	A_1	46	152	4·2	.	29
		S_2	10·8 + 27	1481 − 4	259	140	+	A_2	49	143	5·7	21	.
mean ± S.E.		S_1	9·0 ± 2·1	1733 ± 148	286 ± 35	173 ± 4·7	.	A_1	57 ± 2·6	156 ± 4·4	4·0 ± 0·2	.	.
		S_2	9·1 ± 1·7	1543 ± 180	228 ± 39	121 ± 7·1	.	A_2	54 ± 2·1	154 ± 4·8	4·6 ± 0·3	22 ± 1·3	.
combined mean ± S.E. of all dogs		S_1	8·0 ± 1·2	1701 ± 96	262 ± 29	168 ± 3·8	.	A_1	54 ± 1·8	155 ± 3·1	4·1 ± 0·1	.	.
		S_2	10·4 ± 1·2	1581 ± 145	198 ± 27	111 ± 5·4	.	A_2	52 ± 1·4	154 ± 3·3	4·7 ± 0·2	22 ± 0·1	.

There was, however, an interesting difference between nephrectomized and control dogs (figure 4). Whereas in the controls, prolongation of the interval between adrenal vein cannulation and collection of adrenal blood from 30 min to about 3 h did not affect aldosterone secretion, it increased glucocorticoid secretion. In contrast, aldosterone secretion of nephrectomized dogs was high soon after the nephrectomy, and fell during the waiting

period. These results indicate that the long 'rest' after surgery is, in fact, a stress, as judged from the high glucocorticoid secretion; furthermore, that the rise in aldosterone secretion shortly after nephrectomy might indeed be due to a release of renin during excision of the kidney; as the circulating renin is gradually disposed of, its effect on aldosterone secretion disappears during the long waiting period.

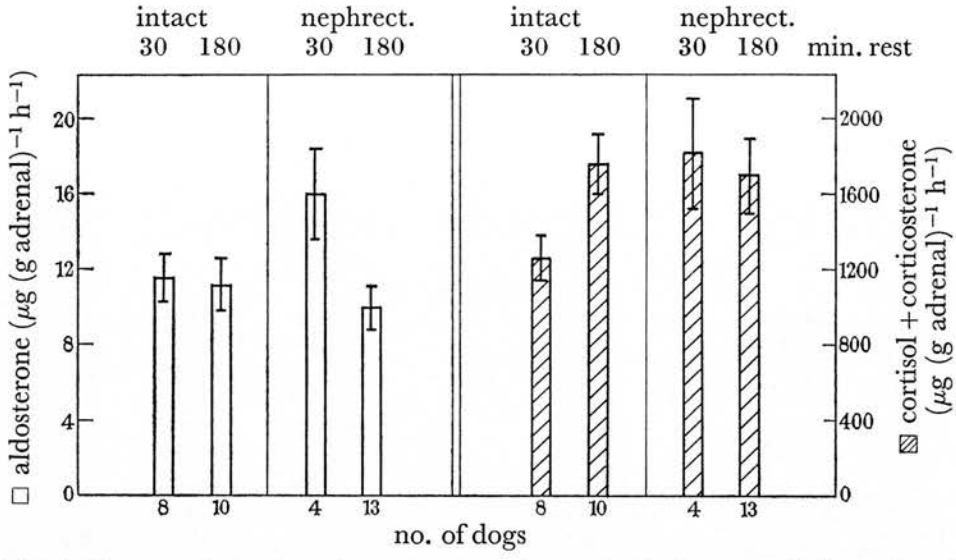

FIGURE 4. The influence of the duration of the resting period after completion of surgery on the secretion rates of corticosteroids (means ± S.E.) in control dogs and nephrectomized dogs. (Aldosterone figures corrected for losses.)

Conclusions

The experiments on nephrectomized dogs have shown that the kidneys are not essential for the response of aldosterone secretion to haemorrhage in dogs with intact pituitary glands. Assuming that renin and *ACTH* are the most potent stimuli involved in this mechanism, the results on hypophysectomized and on nephrectomized dogs provide evidence that the two agents can replace each other. If this assumption is correct, the rise in aldosterone secretion observed in nephrectomized dogs indicates that the pituitary gland is able to increase its *ACTH* output under conditions under which glucocorticoid secretion is already maximal.

IV. ALDOSTERONE SECRETION AFTER HAEMORRHAGE IN HYPOPHYSECTOMIZED-NEPHRECTOMIZED DOGS

The results of the previous sections could also have been obtained if renin and *ACTH* were not the only stimuli for aldosterone secretion called into action by haemorrhage. The actions of these two substances could be supplementary to that of other factors. As discussed on p. 289, this possibility was tested by Ganong & Mulrow (1961, 1962) and by Davis, Carpenter, Ayers, Holman & Bahn (1961) who found no evidence for it. Their experiments were conducted on hypophysectomized-nephrectomized dogs in which secretion rates of all adrenal steroids were extremely low. Thus the question arose, whether in the

absence of these organs, particularly through lack of *ACTH*, steroid synthesis was inhibited to a degree that it could not be accelerated by any stimulus. Experiments were therefore carried out on hypophysectomized-nephrectomized dogs in which steroid synthesis was maintained by a continuous infusion of *ACTH*. The rate was kept below that at which *ACTH* would be expected to be released during operative stress.

Procedures and observations

Dogs infused with ACTH *alone or with noradrenaline and* ACTH

In all animals the right kidney was removed under sodium pentobarbitone anaesthesia and aseptic conditions 10 to 61 days before the acute experiment. On the day of the experiment the pituitary was extirpated first, and then the left kidney. Adrenal blood collection was begun 3 h after nephrectomy and 40 min after starting an infusion of *ACTH* (0·01 or 0·04 m-u. min^{-1} kg^{-1}) which was continued throughout the experiment. For the first 10 min infusion rate was double that indicated. In some dogs L-noradrenaline bitartrate was infused into the right jugular vein. Table 12 summarizes the results of experiments in which the infusion rate of *ACTH* min^{-1} kg^{-1} was 0·01 m-u.; tables 13 and 14 those in which it was 0·04 m-u. Noradrenaline was infused in the experiments of table 14.

TABLE 12. EFFECT OF HAEMORRHAGE ON CORTICOSTEROID SECRETION IN HYPOPHYSECTOMIZED-NEPHRECTOMIZED DOGS, MAINTAINED ON A CONTINUOUS I.V. INFUSION OF 0·01 m-u. *ACTH* min^{-1} kg^{-1} (0·02 FOR THE FIRST 10 min)

Right nephrectomy 12 to 19 days, hypophysectomy 3½ h, left nephrectomy 3 h, start of *ACTH* infusion 40 min before collection of first adrenal blood sample (S_1); second sample (S_2) immediately after S_1, third sample (S_3) after haemorrhage. Arterial blood samples taken at start of S_1 (A_1), at end of haemorrhage (A_2) and at end of S_3 (A_3). Left flank incision, adrenal blood collection periods 30 to 40 min. (Aldosterone figures corrected for losses.)

dog no.	body wt. (kg)	adrenal blood sample no.	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change) aldosterone		cortisol+corticosterone		adrenal blood flow (ml./h)	mean b.p. (mmHg)	Mayer waves	arterial blood samples	Ht (% red cells)	plasma conc. (m-equiv./l.) Na^+	plasma conc. (m-equiv./l.) K^+	blood withdrawn before S_3 (ml./kg)	100 m-equiv. Na^+/day for (no. of days)
Circulation satisfactory															
364,* male	20·0	S_1	(2·2)*	(−5)*	(424)*	(−78)*	190	140	−	A_1	58	164	3·7	.	135
		S_2	2·3	−9	92	+7	176	145	−	A_2	55	161	4·1	.	.
		S_3	2·1		98		130	95	+	A_3	56	156	4·3	17	.
Circulation unsatisfactory															
361,† female	12·5	S_1	14·5		475		128	125	−	A_1	47	155	4·3	.	99
		S_2	14·5	0	264	−45	152	140	−	A_2	47	159	4·8	.	.
		S_3	9·2	−37	307	+16	106	85	+	A_3	45	148	5·1	4·1	.
362, female	9·4	S_1	12·6		137		138	118	−	A_1	41	155	3·7	.	100
		S_2	15·1	+20	206	+51	128	125	−	A_2	50	153	5·0	.	.
		S_3	3·5	−77	148	−28	93	63	−	A_3	47	145	4·9	9·6	.

* *ACTH* infusion for 30 min before S_1 erroneously 0·05 m-u. min^{-1} kg^{-1}.
† Had suffered from diarrhoea after first nephrectomy.

With one exception only (dog 392, table 14), aldosterone secretion after haemorrhage fell, or, at most, remained unchanged. Similarly, in 14 of the 21 dogs, glucocorticoid secretion fell by amounts ranging from 28 to 67 %. Signs of circulatory failure during withdrawal of blood were frequent, and a glance at the tables shows that removal of the usual

TABLE 13. EFFECT OF HAEMORRHAGE ON CORTICOSTEROID SECRETION IN HYPOPHYSECTOMIZED-NEPHRECTOMIZED DOGS, MAINTAINED ON A CONTINUOUS INTRAVENOUS INFUSION OF 0·04 m-u. *ACTH* min^{-1} (kg BODY WEIGHT)$^{-1}$ (0·08 FOR THE FIRST 10 min)

Right nephrectomy 10 to 61 days, hypophysectomy 3½ h, left nephrectomy 3 h, start of *ACTH* infusion 40 min before collection of first adrenal blood sample (S_1). Second sample (S_2) immediately after S_1, third sample (S_3) after haemorrhage. Arterial blood samples taken at start of S_1 (A_1), at end of haemorrhage (A_2) and at end of S_3 (A_3). Left flank incision, adrenal blood collection periods 25 to 40 min. (Aldosterone figures corrected for losses.)

dog no.	body wt. (kg)	adrenal blood sample no.	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change): aldosterone	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change): cortisol + corticosterone	adrenal blood flow (ml./h and % change)	mean b.p. (mmHg)	Mayer waves	arterial blood samples	Ht (% red cells)	plasma conc. (m-equiv./l.): Na^+	plasma conc. (m-equiv./l.): K^+	blood withdrawn before S_3 (ml./kg)	100 m-equiv. Na^+/day (no. of days)
Group I. Circulation satisfactory													
373, male	19·3	S_1	9·2	1802	184 .	140	–	A_1	52	150	3·9	.	30
		S_2	13·4 +46	2052 +14	159	135	–	A_2	53	150	3·6	.	.
		S_3	7·3 −45	1687 −18	112 −30	90	–	A_3	51	148	4·1	15·1	.
374, male	13·0	S_1	10·3	1058	425 .	165	–	A_1	58	152	2·9	.	38
		S_2	13·1 +27	1261 +19	360	175	–	A_2	54	146	2·7	.	.
		S_3	7·8 −40	781 −38	218 −39	90	–	A_3	49	148	2·8	24·6	.
377, male	15·8	S_1	9·2	1759	248 .	140	–	A_1	47	138	4·0	.	30
		S_2	9·5 + 3	2267 +29	336	130	–	A_2	50	130	4·0	.	.
		S_3	7·0 −26	1610 −29	196 −42	80	+	A_3	53	133	3·7	15·5	.
380, female	10·0	S_1	12·3	2417	254 .	143	–	A_1	60	152	4·0	.	29
		S_2	7·0 −43	1917 −21	264	155	–	A_2	55	140	3·7	.	.
		S_3	3·4 −51	742 −61	168 −36	78	–	A_3	51	140	4·0	16	.
mean ± S.E.		S_1	10·3 ± 1·0	1759 ± 364	278 ± 67	147 ± 8	.	.	.	.	.	.	32 ± 2·1
		S_2	10·8 ± 2·0	1874 ± 284	266 ± 51	149 ± 14	.	.	.	.	.	.	.
		S_3	6·4 ± 1·3	1205 ± 336	174 ± 30	85 ± 4	.	.	.	.	.	17·8 ± 3	.
Group II. Circulation unsatisfactory													
366,* male	12·5	S_1	27·3	1492	110 .	115	+	A_1	51	149	5·3	.	64
		S_2	18·0 −34	1367 − 8	100	130	+	A_2	52	146	4·8	.	.
		S_3	9·7 −46	733 −46	76 −24	78	–	A_3	53	152	5·2	12·8	.
369, female	11·5	S_1	8·9	902	140 .	140	–	A_1	50	148	2·4	.	43
		S_2	7·6 −15	1016 +13	188	150	–	A_2	51	147	2·3	.	.
		S_3	6·6 −13	857 −16	90 −62	75	+	A_3	52	150	2·2	10·4	.
372,* male	16·0	S_1	5·8	1374	312 .	120	–	A_1	60	152	3·4	.	36
		S_2	8·6 +48	1336 − 3	348	105	–	A_2	57	.	.	.	.
		S_3	4·6 −47	713 −47	149 −57	70	–	A_3	54	143	3·0	14	.
375,* female	12·5	S_1	13·4	1373	154 .	100	–	A_1	53	127	3·8	.	45
		S_2	12·2 − 9	1380 + 0·5	102	110	–	A_2	53	130	4·2	.	.
		S_3	11·3 − 7	973 −30	102 0	60	–	A_3	49	131	3·8	5·0	.
383, female	13·8	S_1	2·9	269	114 .	125	+	A_1	43	151	2·9	.	91
		S_2	3·6 +24	367 +36	172	120	–	A_2	43	150	3·4	.	.
		S_3	1·6 −56	246 −32	64 −63	60	–	A_3	38	151	3·3	5·4	.
384, female	14·0	S_1	12·3	531	188 .	100	–	A_1	52	159	3·4	.	156
		S_2	10·8 −12	523 − 2	188	130	–	A_2	54	151	4·1	.	.
		S_3	10·0 − 7	249 −53	98 −48	50	–	A_3	51	151	4·1	0	.
385, female	15·3	S_1	18·9	1346	269 .	130	–	A_1	55	163	3·4	.	155
		S_2	12·6 −33	944 −31	283	115	–	A_2	54	156	3·3	.	.
		S_3	4·7 −63	362 −62	77 −73	50	–	A_3	49	159	3·2	8·2	.
mean ± S.E.		S_1	12·8 ± 3·1	1041 ± 182	184 ± 30	119 ± 5·6	.	.	.	.	.	.	84 + 19·6
		S_2	10·5 ± 1·7	990 ± 156	197 ± 34	123 ± 5·7	.	.	.	.	.	.	.
		S_3	6·9 ± 1·3	590 ± 113	94 ± 11	63 ± 4·2	.	.	.	.	.	8·0 ± 1·9	.
groups I and II combined. mean ± S.E.		S_1	11·9 ± 2·0	1302 ± 182	218 ± 29	129 ± 5·9	.	A_1	53 ± 1·6	149 ± 2·9	3·6 ± 0·2	.	65 ± 14·5
		S_2	10·6 ± 1·2	1312 ± 180	227 ± 29	132 ± 6·3	.	A_2	52 ± 1·1	145 ± 2·8	3·6 ± 0·2	.	.
		S_3	6·7 ± 0·9	814 ± 144	123 ± 16	71 ± 4·3	.	A_3	50 ± 1·3	145 ± 2·6	3·6 ± 0·2	11·5 ± 2·0	.

* Suffered from enteritis after the first nephrectomy.

amount of blood was tolerated only once, and that the mean blood pressure after even small haemorrhages was often very low. In the tables, the dogs are grouped according to whether or not their circulatory conditions could be regarded as 'satisfactory'; the criterion was whether an amount of blood exceeding 15 ml./kg could be withdrawn without producing signs of haemorrhagic shock or a fall in blood pressure below 70 mmHg. In both categories, haemorrhage caused similar falls in aldosterone and glucocorticoid secretion and in adrenal blood flow.

TABLE 14. USE OF VASOCONSTRICTORS I: NORADRENALINE

Procedure as in experiments of table 13, but an infusion of L-noradrenaline (0·13 to 0·49 μg min^{-1} (kg body weight)$^{-1}$ begun 1 h before the start of the infusion of *ACTH* and continued throughout the experiment.

dog no.	body wt. (kg)	adrenal blood sample no.	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change): aldosterone	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change): cortisol + corticosterone	adrenal blood flow (ml./h and % change)	mean b.p. (mmHg)	Mayer waves	arterial blood samples	Ht (% red cells)	plasma conc. (m-equiv./l.): Na^+	plasma conc. (m-equiv./l.): K^+	blood withdrawn before S_3 (ml./kg)	100 m-equiv. Na^+/day (no. of days)
Group I. Circulation satisfactory													
376, male	12·3	S_1	6·9	733	112	155	−	A_1	44	153	3·8	.	72
		S_2	6·7 − 3	1333 + 82	120	155	−	A_2	48	151	3·6	.	.
		S_3	5·3 − 21	1163 − 13	134 + 12	100	−	A_3	47	153	3·2	17·9	.
382, male	18·9	S_1	13·6	1308	264	143	−	A_1	45	164	3·7	.	92
		S_2	13·4 − 1	1891 + 45	240	148	−	A_2	46	159	4·1	.	.
		S_3	14·2 + 6	1662 − 12	259 + 8	70	+	A_3	46	164	3·8	16·1	.
Group II. Circulation unsatisfactory													
381, female	15·0	S_1	.	.	.	.	−	A_1	55	141	3·8	.	132
		S_2	18·9 − 42	1620 + 7	348 − 17	140	−	A_2	56	128	3·1	.	.
		S_3	11·0	1734	288	70	−	A_3	52	114	2·9	13	.
386, female	11·0	S_1	5·0	622	96	130	−	A_1	53	144	2·9	.	162
		S_2	3·8 − 24	604 − 3	90	140	−	A_2	55	153	3·2	.	.
		S_3	3·5 − 5	201 − 67	30 − 67	55	−	A_3	53	155	3·1	13·7	.
388, male	12·3	S_1	8·6	1451	302	187	−	A_1	57	159	2·4	.	23
		S_2	8·4 − 2	1457 0	360	185	−	A_2	54	154	2·5	.	.
		S_3	5·2 − 38	971 − 33	149 − 59	60	−	A_3	52	155	2·4	12·2	.
389, female	14·5	S_1	6·3	1145	204	135	−	A_1	53	145	3·0	.	18
		S_2	9·1 + 44	960 − 16	165	115	−	A_2	53	150	3·5	.	.
		S_3	4·7 − 48	646 − 33	150 − 9	85	+	A_3	52	142	3·7	10·7	.
392, female	20·0	S_1	2·4	443	216	100	−	A_1	50	153	3·2	.	16
		S_2	2·7 + 13	643 + 45	246	90	+	A_2	52	159	3·0	.	.
		S_3	4·1 + 52	429 − 33	132 − 46	50	−	A_3	47	153	3·2	2·6	.
mean ± S.E.		S_1	5·6 ± 1·3	915 ± 233	205 ± 42	138 ± 18	.	.	.	.	.	.	70 ± 32
		S_2	8·6 ± 2·9	1057 ± 657	242 ± 52	134 ± 16	.	.	.	.	.	.	.
		S_3	5·7 ± 1·4	796 ± 267	150 ± 41 − 38	64 ± 6	.	.	.	.	.	10·4 ± 2·2	.
combined groups I and II. mean ± S.E.		S_1	7·1 ± 1·5	950 ± 166	199 ± 33	142 ± 12	.	A_1	51 ± 1·9	151 ± 3·2	3·3 ± 0·2	.	74 ± 22
		S_2	9·0 ± 2·1	1215 ± 186	224 ± 40	139 ± 11	.	A_2	52 ± 1·4	151 ± 3·9	3·3 ± 0·2	.	.
		S_3	6·9 ± 1·5	972 ± 223	163 ± 33 − 27	70 ± 7	.	A_3	50 ± 1·1	148 ± 6·1	3·2 ± 0·2	12·3 ± 1·9	.

Table 14 illustrates the effect of giving a constant infusion of noradrenaline throughout the greater part of the experiment. The proportion of dogs in which the circulation collapsed was not decreased, but in the two dogs in which it remained satisfactory the fall in adrenal blood flow was prevented. Perhaps as a result of this, secretion of aldosterone and glucocorticoids did not fall appreciably after haemorrhage, but it did not rise either. One dog (392) tolerated a very small blood loss only and yet appeared to secrete 52 % more

aldosterone after 'haemorrhage'; the absolute figures in this instance were, however, too near the threshold of the method of estimation to be trusted.

The poor circulatory conditions prevailing in the majority of these dogs make it difficult to decide whether the failure of aldosterone secretion to respond to haemorrhage was specifically linked with the absence of pituitary and kidneys or whether it was due to the circulatory collapse, as had been observed in recently splanchnotomized dogs (see part I, table 10, p. 262).

It also seemed important to clarify how far the circulatory collapse was due to the absence from the blood stream of substances known to be released during haemorrhage by the missing organs—renin or posterior lobe hormones—and whether administration of these substances would prevent the collapse.

Effect of angiotensin on tolerance to haemorrhage

In the first experiments an attempt was made to replace the missing effect of the kidneys on the circulation by a constant infusion of angiotensin. The general plan was the same as for the dogs of tables 12 to 14. One hour before starting the infusion of *ACTH* (0·01 m-u. min^{-1} kg^{-1}) a constant infusion of angiotensin was begun (0·01 μg min^{-1} kg^{-1}) which lasted to the end of the experiment. Table 15 shows that in two of the six dogs circulatory conditions were, in fact, very good: initial blood pressure was high and remained at 80 or 90 mmHg after blood loss of 20 ml./kg. Circulation in the remaining experiments, however, was not in the least improved. Reduction in adrenal blood flow after haemorrhage was not consistently lessened by the infusion.

One effect of angiotensin seen in all dogs was the absence of any severe falls in aldosterone secretion after haemorrhage, whereas glucocorticoid secretion was seriously reduced in five dogs. The fact that glucocorticoid secretion was not protected, even in the two dogs in which circulation was good throughout, indicates that the aldosterone secretion was kept high not as a result of improvement in the circulation but by the direct stimulatory action of angiotensin on aldosterone production.

The good circulatory condition of only two out of six similarly treated dogs need not mean that release of renin does not, under natural conditions, help the circulation during haemorrhage. It may be that a constant infusion of angiotensin does not achieve the support of a collapsing circulation which may be achieved by a sudden change in angiotensin concentration, such as one would expect to be produced by an escape of renin from the kidney during haemorrhage.

Effect of posterior lobe hormones on tolerance to haemorrhage

In these experiments infusion of hormones from the posterior lobe of the pituitary was substituted for the release of these hormones during haemorrhage and the effect on the state of the circulation observed. Whereas there is a direct effect of angiotensin on aldosterone secretion, no such effect is established for small doses of vasopressin and oxytocin. Therefore these hormones offer the advantage that their infusion rate can be increased during haemorrhage, as would happen normally, without producing direct effects on synthesis of corticoids.

The experimental plan was similar to that of the previous section, but in place of

angiotensin, infundin ('Infundin', Burroughs Wellcome and Co., a posterior lobe extract containing 10 u./ml.) was infused at rates which are reported (Lauson 1960) to produce nearly maximal antidiuresis in hydrated dogs. This extract was preferred to pure vasopressin for two reasons: it has been shown (Brooks & Pickford 1958) that vasopressin has very different

TABLE 15. USE OF VASOCONSTRICTORS II: ANGIOTENSIN

Procedure as in experiments of table 13, but *ACTH* infusion reduced to 0·01 m-u. min^{-1} kg^{-1} (0·02 m-u. during first 10 min) and intravenous infusion of angiotensin II, 0·01 μg min^{-1} kg^{-1}, begun 1 h before the start of the infusion of *ACTH* and continued throughout the experiment.

dog no.	body wt. (kg)	adrenal blood sample no.	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change): aldosterone	adrenocortical secretion rates: cortisol + corticosterone	adrenal blood flow (ml./h and % change)	mean b.p. (mmHg)	Mayer waves	arterial blood samples	Ht (% red cells)	plasma conc. (m-equiv./l.) Na^+	plasma conc. (m-equiv./l.) K^+	blood withdrawn before S_3 (ml./kg)	100 m-equiv. Na^+/day (no. of days)
Group I. Circulation satisfactory													
390, female	8·8	S_1	17·7	247	81	160	+	A_1	48	131	3·3	.	45
		S_2	15·7 −11	108 − 56	56 −31	170	+	A_2	50	130	4·2	.	.
		S_3	12·3 −22	57 − 47	30 −46	80	+	A_3	34	138	4·4	20	.
400, female	12·0	S_1	10·3	244	102	150	+	A_1	48	154	3·7	.	22
		S_2	9·2 −11	183 − 25	60 −41	155	−	A_2	49	157	4·6	.	.
		S_3	7·9 −14	107 − 42	92 +53	90	+	A_3	47	152	3·8	20	.
mean		S_1	14·0	246	92	155	.	A_1	48	143	3·5	.	.
		S_2	12·5	146	58	163	.	A_2	50	144	4·4	.	.
		S_3	10·1	82	61	85	.	A_3	41	145	4·1	20	.
Group II. Circulation unsatisfactory													
394, male	11·8	S_1	12·7	217	132	95	+	A_1	44	142	3·4	.	43
		S_2	11·5 − 9	443 +104	110 −17	110	+	A_2	45	145	5·0	.	.
		S_3	11·1 − 3	202 − 54	124 +13	60	+	A_3	54	151	4·6	8† (19)	.
396, female	11·3	S_1	9·8	150	116	115	+	A_1	46	153	3·6	.	42
		S_2	15·3 +56	127 − 15	100 −14	125	+	A_2	48	145	4·3	.	.
		S_3	17·5 +14	155 + 22	130 +30	65	+	A_3	47	148	4·1	5† (20)	.
397,* female	12·3	S_1	30·8	236	139	115	+	A_1	48	153	3·6	.	36
		S_2	19·8 −36	258 + 9	137 − 1	115	+	A_2	55	151	4·0	.	.
		S_3	23·8 +20	191 − 26	122 −11	55	+	A_3	55	145	4·3	0† (8)	.
398, female	14·3	S_1	14·6	679	144	145	−	A_1	56	147	3·0	.	13
		S_2	13·9 − 5	428 − 37	138 − 4	150	−	A_2	48	152	3·5	.	.
		S_3	13·0 − 6	240 − 44	96 −30	65	+	A_3	52	147	3·5	5† (12)	.
mean of group II ± S.E.		S_1	17·0 ± 4·7	321 ± 121	133 ± 6·0	118 ± 10·0	.	A_1	49 ± 3	149 ± 3	3·4 ± 0·1	.	.
		S_2	15·1 ± 1·7	314 ± 75	121 ± 9·5	125 ± 8·8	.	A_2	49 ± 2	148 ± 2	4·2 ± 0·3	.	.
		S_3	16·4 ± 2·8	197 ± 17	118 ± 7·4	61 ± 2·4	.	A_3	52 ± 2	148 ± 1	4·1 ± 0·2	.	.
groups I and II combined, mean ± S.E.		S_1	16·0 ± 3·2	296 ± 78	119 ± 9·9	130 ± 10·0	.	A_1	48 ± 2	147 ± 4	3·4 ± 0·1	.	.
		S_2	14·2 ± 1·5	258 ± 60	100 ± 14·7	138 ± 9·8	.	A_2	49 ± 1	147 ± 4	4·3 ± 0·1	.	.
		S_3	14·3 ± 2·3	159 ± 27	99 ± 15·1	69 ± 5·3	.	A_3	48 ± 3	147 ± 2	4·1 ± 0·2	.	.

* Enteritis and sinusitis.

† Amount of blood initially withdrawn in parentheses; abrupt falls in blood pressure necessitated re-infusion, so that final blood removed amounted to the figures shown outside the parentheses.

effects in the presence of oxytocin than when administered on its own, and that most physiological stimuli appear to release both hormones simultaneously (Harris 1955; Baird & Pickford 1958; Pickford 1960). There is, however, no evidence that, during haemorrhage, oxytocin is in fact released along with vasopressin.

In the experiments listed in table 16, a control sample was taken before infundin infusion was started at a rate of 0·036 m-u. min^{-1} kg^{-1}. After 10 min the amount was decreased to

0·009 m-u. min^{-1} kg^{-1} and kept at this speed during the collection of a second adrenal blood sample. During the next 10 min blood was withdrawn and simultaneously the infusion of infundin speeded up fourfold. After the bleeding the slow infusion rate was resumed. In the experiments of table 17 infundin infusion (0·009 m-u. min^{-1} kg^{-1}) was much more prolonged. It was started 1 h before collection of two consecutive samples. Then the dog was bled and during this brief period infusion of infundin was increased fourfold, to be reduced to the original level for the remainder of the experiment.

TABLE 16. USE OF VASOCONSTRICTORS III: POSTERIOR LOBE EXTRACT, SHORT INFUSIONS

Effect of posterior lobe extract ('Infundin', Burroughs Wellcome and Co.) and of haemorrhage on corticosteroid secretion in hypophysectomized-nephrectomized dogs infused with *ACTH*, 0·01 m-u./min/kg body weight (0·02 for the first 10 min). Right nephrectomy 18 days, left nephrectomy 3 h, hypophysectomy 2½ h and start of *ACTH* infusion 40 min before start of collection of first adrenal blood sample. Daily sodium intake 100 m-equiv. for 3 weeks. (Aldosterone figures corrected for losses.)

dog no.	body wt. (kg)	time (min)	adrenal blood sample no.	infusion of infundin (m-u. min^{-1} kg^{-1})	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change)		adrenal blood flow (ml./h and % change)	mean b.p. (mmHg)	Mayer waves	blood withdrawn (ml./kg)
					aldosterone	cortisol+ corticosterone				
422, male	9·5	0– 40	S_1	0	4·6	449	171	150	–	.
		40– 50	none	0·036	· + 4	· −24	· −33	150	–	.
		50– 55	none	0·009	.	.	.	155	–	.
		55– 97	S_2	0·009	4·8	343	114	160	–	.
		99–109	haemorrhage	0·036	· −33	· −50	· −34	140	+	20
		109–114	none	0·009	.	.	.	140	+	.
		114–154	S_3	0·009	3·2	170	87	90	+	.
423, male	14·0	0– 30	S_1	0	3·3	142	190	150	–	.
		30– 40	none	0·036	· 0	· −45	· −16	155	–	.
		40– 45	none	0·009	.	.	.	160	–	.
		45– 75	S_2	0·009	3·3	78	160	170	–	.
		75– 88	haemorrhage	0·036	· −55	· −23	· −27	160	+	19·3
		88– 91	none	0·009	.	.	.	160	+	.
		91–121	S_3	0·009	1·5	61	116	105	+	.
mean			S_1		4·0	296	181	150	.	.
					.	.	.	152	.	.
					.	.	.	158	.	.
			S_2		4·1	211	137	165	.	.
					.	.	.	150	.	19·7
					.	.	.	150	.	.
			S_3		2·4	116	102	98	.	.

From tables 16 and 17 it can be seen that infundin maintained satisfactory circulatory conditions in four out of five dogs, but adrenal blood flow decreased after haemorrhage. No pressor effect of infundin was noticeable at the concentrations used, and the blood pressure fell somewhat during bleeding in spite of the accelerated rate of infusion. However, the falls were less abrupt, and there is no doubt that the infusions exerted a blood pressure maintaining influence.

The short infusion periods of infundin (table 16) did not alter aldosterone secretion during the control period (S_2), and failed to prevent the fall induced by bleeding. In contrast, prolonged infusion (table 17) stimulated aldosterone secretion before, and prevented any appreciable fall after haemorrhage. Production of glucocorticoids was less influenced than that of aldosterone: there was one rise during a pre-haemorrhage period, but no check to the severe falls after bleeding.

In one experiment, vasopressin (0·016 m-u. min^{-1} kg^{-1}) was used instead of infundin. The infusion was started 40 min before taking a control sample and was not speeded up

during the bleeding period. There was circulatory collapse, so that the object of the experiment was not achieved, but an interesting feature was that after 'haemorrhage' (amount of blood withdrawn was 6 ml./kg only) the adrenal blood flow rose and the glucocorticoid secretion did not fall. This observation confirms the striking dependence on blood flow of glucocorticoid secretion in nephrectomized-hypophysectomized dogs, a relation which does not exist in the normal dog except in extreme conditions.

TABLE 17. USE OF VASOCONSTRICTORS III: POSTERIOR LOBE EXTRACT, PROLONGED INFUSIONS

Experimental procedures as in table 16, but Infundin infusion (0·009 m-u. min^{-1} kg^{-1}) started 1 h before the first adrenal blood collection. Rate of infusion quadrupled during the time of blood withdrawal (10 min). Right nephrectomy 5 months previously. Daily sodium intake 100 m-equiv. for 3 months followed by 30 m-equiv. for 6 weeks, and 100 m-equiv./day for the last 2 weeks. Dogs are litter-mates.

dog no.	body wt. (kg)	adrenal blood sample no.	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change): aldosterone	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change): cortisol + corticosterone	adrenal blood flow (ml./h and % change)	mean b.p. (mmHg)	Mayer waves	blood withdrawn before last sample (ml./kg)
416, female	20·0	S_1	3·4	105	132	145	+	.
		S_2	4·5 +32	159 +51	188 +42	150	+	.
		S_3	4·6 + 2	37 −77	108 −43	72	−	10(14)*
417, male	15·6	S_1	3·5	129	116	140	−	.
		S_2	5·9 +69	93 −28	81 −30	145	−	.
		S_3	5·7 − 3	52 −44	76 − 6	120	+	19
421, female	24·0	S_1	3·8	259	209	177	−	.
		S_2	4·2 +11	200 −23	249 +19	180	−	.
		S_3	3·0 −29	86 −57	158 −37	80	−	17
mean ± S.E.		S_1	3·6 ± 0·12	164 ± 48	152 ± 29	154 ± 12	.	.
		S_2	4·9 ± 0·52	151 ± 31	173 ± 49	158 ± 11	.	.
		S_3	4·4 ± 0·80	58 ± 14	114 ± 24	91 ± 15	.	15 ± 3

* Explanation see table 15†.

Effect of blood obtained from dogs with intact kidneys on aldosterone secretion

One unexpected feature of the experiments on hypophysectomized-nephrectomized dogs receiving constant *ACTH* infusions was the great variation between individual dogs of the aldosterone secretion during the control periods. One reason for these variations could be that the response to *ACTH* is influenced by the degree to which renin has previously participated in keeping the rate of aldosterone secretion high. It may be significant in this connexion that two dogs (366, 397, tables 13 and 15) which were suffering from diarrhoea prior to the experiment, had the very high initial aldosterone secretion rates of 27 and 31 μg (g adrenal)$^{-1}$ h^{-1}.

Another factor which could contribute to variations is the replacement of lost blood by donor blood obtained from animals with intact kidneys and pituitary glands. It is well established, that *ACTH* is destroyed quickly in dog's blood on standing (Nelson & Hume 1955), but renin and angiotensin are probably more stable. It is possible that the concentration of these substances in blood taken from dogs with intact kidneys remains high enough, even after storage, to stimulate aldosterone secretion in nephrectomized dogs. However, of two consecutive control samples of adrenal blood collected during constant infusion of

ACTH in nephrectomized-hypophysectomized dogs, the second sample only rarely contained more aldosterone than the first in spite of the increasing amounts of donor blood infused; for example, in table 14, there was only one significant increase in dog 389, and this dog had, in fact, received per kg body weight less donor blood by the end of the second sample than most dogs of the same group.

In order to obtain conditions favourable for the demonstration of an influence on aldosterone secretion of any renin which might be present in donor blood, experiments were carried out in which, in contrast to previous procedures, very large amounts of donor

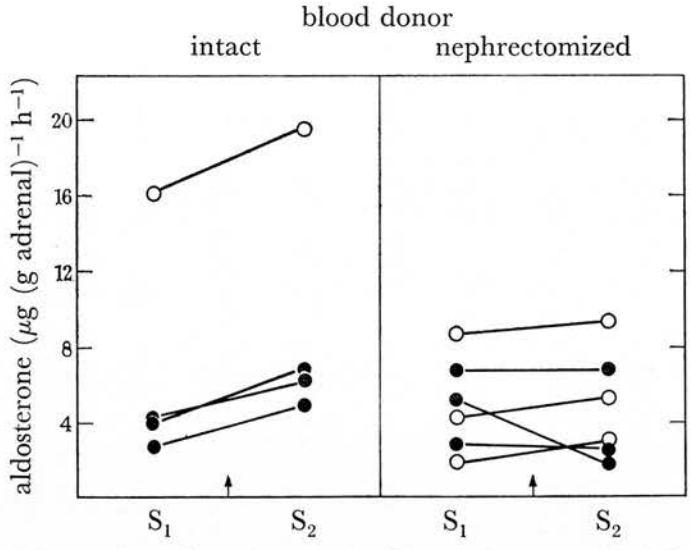

FIGURE 5. Aldosterone secretion of nephrectomized-hypophysectomized dogs given large volumes (20 ml./kg body wt.) of donor blood. Comparison of infusion of donor blood from intact or from nephrectomized dogs. The donor blood was given rapidly between the collection of a first (S_1) and a second (S_2) adrenal blood sample. *ACTH* was infused at rates of 0·01 (●) or 0·02 (○) m-u. min^{-1} kg^{-1} body wt. throughout the experiment (see also tables 18 and 19).

blood were infused during a short period. Blood taken from dogs with intact kidneys or from dogs nephrectomized under chloralose 3 h before bleeding was infused into nephrectomized-hypophysectomized dogs given a constant infusion of *ACTH*. A control sample of adrenal blood was collected, then donor blood 20 ml./kg infused within 10 min and a second adrenal blood sample taken. The blood lost during the collection periods was also replaced by the appropriate donor blood. The results are summarized in figure 5. The dogs given blood from dogs with intact kidneys responded with rises in aldosterone secretion. Among the dogs receiving blood from nephrectomized donors significant rises did not occur. These experiments provide evidence for the presence, in blood stored at 4 °C for 1 to 4 days, of a substance which stimulates aldosterone secretion and which is not evident in blood taken 3 h after removal of both kidneys. In all probability this substance is renin released during haemorrhage. Its stimulant effect is not evident unless large amounts of donor blood are rapidly infused, but its lack might have contributed to the severe falls in aldosterone secretion after bleeding seen in tables 12 to 17 and coinciding with cessation of infusion of donor blood.

Effect of expansion and subsequent reduction of the circulating blood volume on aldosterone secretion

In the previous sections it was shown that the circulation of nephrectomized-hypophysectomized dogs maintained on *ACTH* infusions was so labile that bleeding was not tolerated unless vasoconstrictor substances were infused. It was hoped that haemorrhage might be tolerated without the need of supporting therapy if the blood was withdrawn after a preliminary expansion of the blood volume. This was tried on the dogs used for the experiments illustrated in figure 5 by bleeding them after the collection of the second sample of

TABLE 18. EFFECT OF EXPANSION AND REDUCTION OF THE BLOOD VOLUME ON CORTICOSTEROID SECRETION IN HYPOPHYSECTOMIZED-NEPHRECTOMIZED DOGS MAINTAINED ON *ACTH*. DONOR BLOOD OBTAINED 1 TO 4 DAYS PREVIOUSLY FROM DOGS WITH INTACT KIDNEYS

Experimental details: right nephrectomy 3 to 5 months, left nephrectomy 3 h, hypophysectomy 2½ h, start of *ACTH* infusion 40 min before collection of first adrenal blood sample (S_1), S_2 after expansion, S_3 after reduction of blood volume. Left flank incision, aldosterone figures corrected for losses, all dogs litter-mates of dogs from table 17.

dog no.	body wt. (kg)	adrenal blood sample no.	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change): aldosterone	adrenocortical secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change): cortisol+corticosterone	adrenal blood flow (ml./h and % change)	mean b.p. (mmHg)	Mayer waves	change in blood volume (ml./kg)	donor blood infused during collection periods (ml./kg)	*ACTH* infusion (m-u. min^{-1} kg^{-1})	daily Na$^+$ intake (m-equiv.)
418, male	16·5	S_1	2·6	167	180	170–80*	+		7·9		
		S_2	4·9 +88	95 −43	174 −3	50–90	−	+20	10·3		
		S_3	3·6 −27	43 −55	72 −59	60–80	−	−21	.	0·01 (0·02 for the first 10 min)	100 for 100 days, then 30 for 31 days, then 100 for 8–18 days
419, female	17·5	S_1	4·0	423	240	140	−		7·4		
		S_2	6·2 +55	612 +45	260 +8	160	−	+20	11·7		
		S_3	1·9 −69	243 −60	212 −18	95	−	−17	.		
420, male	15·3	S_1	3·9	404	290	130	−		9·9		
		S_2	6·8 +74	486 +20	290 0	160	+	+20	9·3		
		S_3	4·0 −41	247 −49	250 −14	80	+	−20	.		
mean ± S.E.		S_1	3·5 ± 0·4	331 ± 82	237 ± 32	135†	·				
		S_2	6·0 ± 0·6	398 ± 156	241 ± 35	160	·	+20 ± 0			
		S_3	3·2 ± 0·6	178 ± 67	178 ± 54	88	·	−19 ± 1·2			
415, male	20·0	S_1	16·1	1004 −18	345	164	−		6·3	0·02 (0·04 for first 10 min)	100 for 100 days
		S_2	19·7 +22	822 −39	432 +25	165	−	+20	7·3		
		S_3	10·6 −46	505	276 −36	147	−	−20	.		

* Donor blood badly tolerated; blood pressure values at beginning and end of respective sample.
† Means of dog 419 and 420.

adrenal blood. At an earlier stage of this work (see figure 2, part I, p. 249) rises in aldosterone secretion had been observed under similar conditions in dogs possessing pituitaries and kidneys. At that time this type of experiment had been abandoned because of the frequent occurrence of incompatibility reactions to foreign blood, no antihistamine drug having been administered. However, in all subsequent work mepyramine was given and this greatly reduded the incidence of reactions to moderate volumes of donor blood. The plan of the experiments was, thus, to take a control sample of adrenal blood, then to infuse donor blood, 20 ml./kg, over a period of 10 min and to start collecting a second sample 5 min later. After this the dog was bled to remove the surplus blood volume and a third sample was taken. Table 18 shows the results when donor blood from dogs with intact kidneys was used, and table 19 those obtained with nephrectomized donors. The dogs of table 18 all responded to infusion of, presumably, renin-containing blood by an increase in

aldosterone secretion, but after withdrawal of the excess volume, secretion fell to or below the original level. The haemodynamic effect of the withdrawal of blood was as severe as the effect of true haemorrhage in normal dogs.

TABLE 19. PROCEDURE AS IN TABLE 18, BUT DONOR BLOOD OBTAINED FROM DOGS 3 h AFTER NEPHRECTOMY, AND STORED AT 4 °C FOR 1 OR 2 DAYS

100 m-equiv. Na^+ daily for 23 to 43 days, right nephrectomy 20 to 38 days before experiment.

dog no.	body wt. (kg)	adrenal blood sample no.	adrenocorticol secretion rates (μg (g adrenal)$^{-1}$ h^{-1} and % change) aldosterone	cortisol + corticosterone	adrenal blood flow (ml./h and % change)	mean b.p. (mmHg)	Mayer waves	change in blood volume (ml./kg)	donor blood infused during collection periods (ml./kg)	*ACTH* infusion (m-u. min^{-1} kg^{-1})
424,* male	15·3	S_1	5·1	122	105	100	−		6·5	
		S_2	1·8 −65	115 −6	65 −38	160	−	+20	19·6	
		S_3	2·5 +39	92 −20	43 −34	70	−	−16	.	
425, male	16·5	S_1	2·8	226	152	135	−		4·5	
		S_2	2·5 −11	112 −50	152 0	100	−	+20	8·2	
		S_3	3·6 +44	101 −10	132 −13	70	−	−19	.	0·01 (0·02 for the first 10 min)
426,† male	15·5	S_1	.	142	172	120	−		6·5	
		S_2	4·5	130 −8	140 −19	140	+	+20	6·8	
		S_3	1·3 −71	34 −74	69 −51	80	+	−21‡	.	
427, male	15·2	S_1	6·7	146	192	95	−		8·2	
		S_2	6·7 0	111 −24	128 −33	110	−	+20	9·2	
		S_3	8·0 +19	91 −18	102 −20	110	−	−20	.	
mean ± s.e.		S_1	4·9 ± 1·1	159 ± 23	155 ± 19	113 ± 9				
		S_2	3·9 ± 1·1	117 ± 4	121 ± 19	128 ± 14		+20 ± 0		
		S_3	3·9 ± 1·5	80 ± 15	87 ± 19	83 ± 9		−19 ± 1·1		
429, male	11·5	S_1	8·5	431	240	120	−		10·9	
		S_2	9·4 +11	557 +29	184 −23	160	−	+20	13·0	
		S_3	12·4 +32	658 +18	152 −17	95	−	−25	.	
432,§ male	13·4	S_1	1·9	341	226	102	−		9·0	0·02 (0·04 for the first 10 min)
		S_2	3·0 +58	389 +14	238 +5	150	−	+20	11·2	
		S_3	1·5 −50	177 −54	126 −47	75	−	−17	.	
433, female	10·8	S_1	4·2	408	176	110	−		9·3	
		S_2	5·3 +26	369 −10	150 −15	140	−	+20	11·1	
		S_3	4·7 −11	631 +71	180 +20	120	+	−23	.	
mean ± s.e.		S_1	4·9 ± 1·9	393 ± 27	214 ± 19	111 ± 5	.	.	.	.
		S_2	5·9 ± 1·8	438 ± 60	191 ± 26	150 ± 6	.	+20 ± 0	.	.
		S_3	6·2 ± 3·2	489 ± 156	153 ± 16	97 ± 13	.	−22 ± 2·4	.	.

* Heavy wound bleeding during S_2 only (all blood lost simultaneously replaced by donor blood).
† Ht = 70%.
‡ Blood withdrawn between S_2 and S_3 9 ml./kg when b.p. fell to 60 mmHg; 5 min after start of S_3 b.p. recovered and bleeding was resumed. (S_1 and S_2 collected for 30 min, S_3 for 45 min.)
§ No mepyramine given. Dog developed severe urticaria after donor blood infusion.

Of the dogs infused with blood from nephrectomized donors only one (424, table 19) responded to the expansion of the blood volume by a fall in aldosterone secretion such as one would expect to see in a dog possessing kidneys and a pituitary gland. Since no other dog responded in this way no explanation of the result can be attempted. None of the dogs tolerated subsequent withdrawal of blood exceeding the surplus previously infused; restoration of the original blood volume was followed in three animals by small increases in aldosterone secretion and this was significantly above the initial secretion rate only once (dog 429). All aldosterone values were small in absolute terms and small differences therefore unreliable.

Two dogs (426, 432) showed sharp falls in aldosterone secretion after withdrawal of blood; in these animals glucocorticoid secretion and adrenal blood flow were also greatly

diminished, and the reduced secretion of aldosterone can be interpreted as damage due to impaired circulation.

It would, therefore, appear that in those dogs in which adrenal circulation remained satisfactory, withdrawal of surplus blood following initial expansion led to small increments in aldosterone secretion. Yet even with this technique haemorrhage was not tolerated, and the infusion of the large quantities of donor blood needed for the initial expansion of the circulatory volume frequently caused excessive wound bleeding or falls in blood pressure in spite of the antihistamine drug.

Conclusions

Maintenance of the capacity for steroid synthesis in hypophysectomized-nephrectomized dogs by a constant infusion of *ACTH* did not restore the ability of these animals to respond to haemorrhage by a rise in aldosterone secretion. The result was not changed by administering a constant infusion of angiotensin. These observations are compatible with the view that the sudden increase in the blood concentration of *ACTH* and renin, as elicited by blood loss, is the essential stimulus for aldosterone secretion after haemorrhage.

The interpretation of experiments on acutely hypophysectomized dogs without kidneys was complicated by the extreme lability of the circulation. Many experiments had to be carried out in order to obtain a sufficient number in which blood loss was tolerated, so that circulatory breakdown could be excluded as cause for the failure of aldosterone secretion to rise after blood loss. Whereas haemorrhage was well tolerated by intact and by nephrectomized dogs, circulatory collapse occurred in 40 % of the hypophysectomized dogs, and in more than 60 % of the dogs in which the kidneys were absent as well. Acute hypophysectomy may cause some damage to hypothalamic centres involved in vasomotor control. More important probably, is the loss of the posterior lobe which is known to release vasopressin in severe haemorrhage.

A blood pressure supporting effect of the posterior pituitary hormones was demonstrated in the experiments with infundin. The doses used were those known to exert a full antidiuretic effect; they were much lower than the peak secretion rate found in dogs after blood withdrawal of approximately 30 ml./kg by Weinstein, Berne & Sachs (1960): for a few minutes, these authors observed a release of vasopressin of the order of 15 m-u. min^{-1} kg^{-1}. However, even the small doses caused a direct stimulation of corticoid, particularly of aldosterone secretion, so that the use of larger doses would have interfered with the object of the experiments. The observation that glucocorticoid secretion was only once enhanced by these low concentrations agrees with the observation (Hilton 1960) that at least 1 m-u./min of vasopressin is required by arterial infusion into the dog adrenal gland to produce acceleration of cortisol secretion.

Severe falls in the secretion rates of all corticosteroids after bleeding was another feature in which hypophysectomized-nephrectomized dogs differed from any other group. Part of the reduction in aldosterone secretion may have been caused by the infusion schedule of donor blood: during the collection of pre-haemorrhage samples, moderate quantities of donor blood which must have contained a little renin were infused to replace the blood lost from the adrenal, whereas no such replacement was made of blood collected after haemorrhage. Though it is doubtful whether the amount of donor blood infused was sufficient to act as a stimulus to aldosterone production, larger quantities were shown to act in this way

(figure 5). Such an explanation can hardly be valid for the fall in glucocorticoids since there was no evidence that infusion of angiotensin enhances glucocorticoid secretion (table 15). Yet, circulatory failure is not the sole explanation of reduced corticoid production after haemorrhage. This follows from the fact that it also occurred when blood pressure and adrenal blood flow remained higher than the critical level at which steroid secretion would be inhibited in intact dogs. Simultaneous absence of the pituitary gland and the kidneys appears to impair the reserve power of the adrenal to supply corticosteroids in adverse conditions.

DISCUSSION

After acute blood loss a release of aldosterone might exert a beneficial effect by preserving salt and water, and its increased secretion under these circumstances can be regarded as a homeostatic mechanism. In the intact organism stimulation of aldosterone secretion after haemorrhage is safeguarded by at least two mechanisms, an increased secretion of *ACTH* and a release of renin. These two factors can replace each other so that neither factor can rightly be regarded as 'the' glomerulotrophic hormone. Thus, in the presence of the pituitary, post-haemorrhage increase in aldosterone secretion was of the same frequency and size whether or not the kidneys were there. This indicated that under our experimental conditions, the influence of *ACTH* on the zona glomerulosa was just as important as that of angiotensin.

In hypophysectomized dogs with intact kidneys, in which a submaximal secretion rate of adrenal steroids was maintained by a constant infusion of *ACTH*, rises in aldosterone and glucocorticoid secretion after bleeding were presumably caused by angiotensin. This interpretation does not seem quite compatible with the observation of Scornik & Paladini (1964) that after bleeding a significant rise in the concentration of angiotensin in arterial blood occurs only after 1 h, much later than the release of aldosterone. However, it is possible that effective amounts of angiotensin reach the adrenal long before any increase in peripheral blood concentration becomes measurable.

In nephrectomized dogs with intact pituitaries the *ACTH* released by bleeding was probably the main stimulus for the adrenal cortex, though a contributory role of vasopressin is possible (see table 17). Glucocorticoid secretion was usually near maximal beforehand, so that the effect was confined to an increase in aldosterone secretion.

Rises of aldosterone secretion after haemorrhage were very rarely seen under circumstances in which aldosterone secretion was already high before bleeding, e.g. in sodium depletion, after severe surgery, or during an attack of enteritis. Dogs in which the circulation collapsed after haemorrhage also frequently failed to show the increase in aldosterone. This may be due to circulatory failure at many sites, possibly to reduced hormone synthesis in the adrenal itself. Furthermore, certain observations suggested that prolonged sodium loading can render the zona glomerulosa insensitive to stimuli. Too little is yet known about the suggested inhibitory effect of extracts from a diencephalic-pineal complex (Farrell 1964) on aldosterone secretion to assess its possible role in certain types of failure.

Although the present experiments have shown that the rise in aldosterone secretion in response to a massive haemorrhage does not depend on angiotensin as the sole stimulus, there is little doubt that the increased aldosterone production observed when the renal circulation is impeded by constriction of the inferior vena cava, or by large aortic-caval

fistulae, or by narrowing of the renal arteries, is caused by excessive renin and disappears when the kidneys are removed. Under these conditions, stimulation of aldosterone production cannot be regarded as a homeostatic mechanism. On the contrary, aldosterone adds to the damage done to the organism by increasing salt and water retention.

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THE PART PLAYED BY ACTH IN DETERMINING THE RATE OF ALDOSTERONE SECRETION DURING OPERATIVE STRESS

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(*Received* 16 *January* 1964)

In order to interpret results of experiments on the control of aldosterone secretion in the dog (Holzbauer & Vogt, 1959, 1960, 1963) information on two questions was needed. First, information was required on the rate of secretion of adrenocorticotrophic hormone (ACTH) in a dog subjected to adrenal vein cannulation in chloralose anaesthesia, and secondly, on the effect of acute hypophysectomy and infusions of ACTH on the production of aldosterone.

The experiments described in this paper answer the second question by direct measurements of aldosterone in adrenal vein blood; the first question, requiring an estimate of ACTH secretion which cannot at present be measured directly, has been dealt with by comparing the rates of production of steroids by the adrenal before hypophysectomy with the rates found after hypophysectomy, during infusions of ACTH. A preliminary report on this work has been published (Holzbauer, 1963).

METHODS

Operative procedures

The experiments were carried out on mongrel dogs of different ages and both sexes. They weighed between 8·8 and 20 kg and were kept in the animal house for 2–52 days. All dogs except no. 334, which was kept for 2 days only, were vaccinated on their admission against distemper, either with non-specific γ-globulin, or with Epivax-plus (Burroughs Wellcome). Their daily ration consisted of 600 g dog biscuits and 300 g fresh meat, 4 g of a yeast extract and 4 g of a sheep-bone extract. This mixture contained approximately 30 m-equiv Na^+ and 65 m-equiv K^+ and was supplemented with a further 70 m-equiv Na^+ in the form of NaCl. Drinking water was unrestricted.

The anaesthesia used was ether followed by chloralose (BDH, 70 mg/kg as 0·67 % solution in 0·9 % NaCl) injected into the femoral vein. In some experiments further doses of 0·2 or 0·3 g of chloralose were required. The dogs were kept warm by an electric heating pad. A tracheal cannula was inserted, avoiding any interference with the blood supply to the thyroid gland. The region of the pituitary gland was exposed through the roof of the mouth. A dental drill was used and great care taken to avoid damage to the dural covering of the gland. A piece of sterispon (absorbable gelatin sponge B.P.) was lightly placed into the

bone cavity and the mouth was closed. During this part of the experiment the dog was put on artificial respiration (Starling pump, 20 rev/min, stroke 15 ml./kg body wt.).

The left adreno-lumbar vein was then exposed by an incision in the flank and a siliconed glass cannula inserted close to the lateral edge of the adrenal gland after accessible tributaries coming from other tissues had been tied. The cannula was connected by a silicone rubber tube to a T-piece, one limb of which would divert the blood flow into a measuring cylinder while the other was connected to a cannula tied into the central end of a femoral vein. After injection of heparin (350 u./kg) and ligation of the adreno-lumbar vein at its junction with the vena cava, the adrenal vein blood was either returned through the femoral vein shunt into the dog or drained into a cylinder standing in ice water. The blood pressure was recorded from the right femoral artery with a mercury manometer on a smoked drum.

Blood losses from adrenal vein blood collection or from wound bleeding were replaced by infusions of dog's blood. Mepyramine maleate, 1 mg/kg, was given intravenously in order to try to antagonize ill effects on the circulation caused by infusions of homologous blood (Bliss, Johns & Burgen, 1959, and Remington & Baker, 1959). The donor blood was obtained from non-hypophysectomized dogs which were exsanguinated under ether anaesthesia from a carotid artery on the day before the experiment. Heparin (6500 u./l.) and in some cases glucose (0·2 g/l.) was added. Glucose was reported to decrease the spontaneous haemolysis which occurs in dog's blood on standing (Swisher & Young, 1961). The blood was kept at +4° C for at least 24 hr, was filtered through gauze before use and warmed to 39° C in a water-bath in which it was kept in a slow rhythmic motion. The donor blood was obtained so far in advance of the experiment in order to ensure complete disappearance of any ACTH it contained. According to Nelson & Hume (1955) little or no ACTH can be found in dog's blood as early as 3 hr after withdrawal from the body.

Half an hour after the shunt flow had been established a sample of adrenal venous blood was collected over a period of 20–30 min. Artificial respiration was then resumed, the dural covering of the pituitary gland split and the gland removed by suction. A piece of sterispon was put in place of the pituitary, the cavity in the bone was filled with bone wax, the mouth closed and the artificial respiration discontinued. Another injection of heparin (150 u./kg) was given and the dog was allowed to rest for 1 hr. After this interval one or two adrenal blood samples were collected. Then ACTH solutions were infused into the left jugular vein at a rate of 0·7 ml./min by means of a Palmer slow infusion apparatus. Usually three concentrations differing by a factor of 10 were used in ascending order. Adrenal vein blood collections were started when the infusions had been running for 5 min and were continued for 5 min beyond the end of each infusion. Usually 1 min elapsed between the end of a collection period and the start of the next infusion. At the end of each experiment the skull was opened and the base of the brain searched for pituitary remnants.

Adrenocorticotrophic hormone

The ACTH used in these experiments was 'cortrophin' (Organon) of porcine origin for intramuscular injections, 1·7 i.u./mg. As the preparation was several years old, its potency was tested against a more recently manufactured batch of 'cortrophin' (Organon) of porcine origin prepared for intravenous use and containing 31 i.u./mg. The comparison was carried out on a hypophysectomized dog according to the method for ACTH assay described by Nelson & Hume (1955). The test showed that the older preparation had not lost activity: the ratio of the stimulating effect of the two batches on the secretion of cortisol and corticosterone in the hypophysectomized dog was about the same as the ratio of the activities given by the manufacturers for intravenous administration. The pressor activity of the preparation used was tested on the blood pressure of the pithed rat. It was found that 100 m-u. of ACTH were equivalent to about 0·8 m-u. of pitressin. The concentrations used for the experiments did not have any pressor effect in the dogs.

Chemical procedures

The basic outlines for the methods used for the estimation of aldosterone, cortisol and corticosterone have been described (Holzbauer & Vogt, 1961). Some modifications were made.

(1) The ethanolic phase obtained after defatting the extracts with petroleum ether was not evaporated in vacuo but was taken to dryness in a water bath at 40° C by means of a stream of compressed air.

(2) The aldosterone region in the first chromatogram was located by its position relative to cortisol. In a number of trial runs the ratio between the distance from the origin of the centre of the cortisol spot and the centre of the aldosterone spot was found to be 0·78. A rectangular piece of paper including a region 4 cm above and below the calculated centre of the aldosterone spot was eluted. In samples collected after hypophysectomy 40 μg cortisol was applied with the extract in order to make the cortisol region visible.

(3) The second chromatogram was run on 4 cm lanes, so that four samples could be applied to each paper.

(4) Washing of the papers used for the third chromatogram was restricted to the second washing for 48 hr in ethyl acetate-methanol.

(5) The chromatograms for the estimation of cortisol and corticosterone were run on papers washed as described under 4.

(6) Elution was often found to be incomplete if the size of the eluted rectangles was large. Therefore periods of 3 hr were allowed for papers 4×9 cm, and at least 4 hr for papers 8×9 cm.

(7) The paper blanks against which the colour formed by the steroids when allowed to react with blue tetrazolium was read were reduced by distilling the petroleum ether and the benzene used in the chromatography. The mean value for the blanks was reduced to the equivalent of 0·35 μg aldosterone (S.D. $\pm$ 0·1).

(8) The cortisol and corticosterone contained in the adrenal blood samples which were collected after hypophysectomy were not estimated by the 'macromethod' (Vogt, 1955) but were allowed to react with the small quantities of reagents used for the aldosterone estimations and the colour was read in the microcells.

(9) The recovery of aldosterone was estimated in each individual sample with the aid of 7-^{3}H-aldosterone (specific activity 20 μc/μg) which was apparently radiochemically pure. A solution containing 0·013 μc in 0·1 ml. of ethanol was prepared and kept at $-14°$ C. Immediately at the end of a period in which adrenal vein blood was collected 0·1 ml. of this solution was added to the blood. Recovery was determined from the tritium counts given by the final solution used for colorimetry. The procedure was as follows: after the aldosterone content of each sample had been estimated by its colour reaction with blue tetrazolium, the solution was transferred quantitatively into a counting vial with the help of 2 ml. ethanol and a Pasteur pipette. When 15 ml. of scintillator (4 g 2,5-diphenyloxazole and 0·1 g 1,4-bis-2-(5-phenyloxazolyl)-benzene in 1 l. toluene) was added a clear solution was obtained. The radio-activity was determined in a TRI-CARB liquid scintillation spectrometer. In order to calculate the recovery, the number of counts obtained from each sample was compared with that obtained from the quantity of 7-^{3}H-aldosterone originally added and measured in the presence of the same chemicals. For this purpose 0·1 ml. of the 7-^{3}H-aldosterone solution and 3 μg of cold aldosterone were pipetted into a counting vial. The volume was made up to 0·45 ml. with 95 % ethanol. Tetraethylammonium hydroxide and blue tetrazolium were added and the vial incubated as if it were a genuine sample. Acetic acid, 2 ml. of ethanol and the scintillator were then added. The number of counts obtained was regarded as 100 % recovery. The presence of ethanol and the other chemicals in the scintillator caused quenching by about 50 %. Variations of the degree of quenching in samples containing different amounts of cold or hot aldosterone or eluates from paper strips of different sizes were found to lie within the range of the counting error. Background counts

TABLE 1. Secretion rates of aldosterone, cortisol and corticosterone during stress, after hypophysectomy and during infusions of ACTH. Observations obtained in 6 representative experiments. S = adrenal blood sample

Dog no.	Body wt. (kg)			S_1 before hypophysectomy	S_2 started 60 min after hypophysectomy	S_3 started 90–120 min after hypophysectomy	S_4 During infusion of ACTH 0·03 (m-u./min/kg body wt.)	S_5 During infusion of ACTH 0·3 (m-u./min/kg body wt.)	S_6 During infusion of ACTH 3·0 (m-u./min/kg body wt.)
331 (female)	10·2	Secretion rates (μg/g adrenal/hr)	Aldosterone	9·5	4·0	2·8	6·7	10·6	11·4
			Cortisol	—	46·0	4·0	762·0	1493·0	1493·0
			Corticosterone	—	61·0	23·0	244·0	435·0	435·0
		Mean blood pressure (mm Hg)		160	130	137	140	140	140
		'Adrenal' blood flow (ml./hr)		180	106	108	103	86	62
334 (female)	16·0	Secretion rates (μg/g adrenal/hr)	Aldosterone	11·9	7·6	3·3	8·8	15·1	22·4
			Cortisol	1228·0	109·0	65·0	573·0	960·0	1189·0
			Corticosterone	436·0	55·0	44·0	218·0	404·0	431·0
		Mean blood pressure (mm Hg)		150	135	135	130	135	130
		'Adrenal' blood flow (ml./hr)		255	276	278	312	274	300
340 (female)	12·8	Secretion rates (μg/g adrenal/hr)	Aldosterone	16·8	1·9	0·6	7·2	20·2	26·3
			Cortisol	1268·0	72·0	27·0	960·0	1467·0	1368·0
			Corticosterone	725·0	22·0	24·0	444·0	797·0	769·0
		Mean blood pressure (mm Hg)		135	123	130	130	132	140
		'Adrenal' blood flow (ml./hr)		130	91	82	82	94	101
341 (female)	16·5	Secretion rates (μg/g adrenal/hr)	Aldosterone	21·3	18·4	21·3	18·4	27·0	26·1
			Cortisol	788·0	69·0	33·0	1216·0	1327·0	1309·0
			Corticosterone	299·0	44·0	48·0	552·0	735·0	713·0
		Mean blood pressure (mm Hg)		165	150	150	148	150	150
		'Adrenal' blood flow (ml./hr)		144	115	110	137	158	182
342 (male)	14·5	Secretion rates (μg/g adrenal/hr)	Aldosterone	14·5	7·1	13·2	18·3	14·7	29·3
			Cortisol	1418·0	106·0	89·0	1227·0	1362·0	1384·0
			Corticosterone	826·0	96·0	82·0	647·0	726·0	949·0
		Mean blood pressure (mm Hg)		140	135	135	140	140	140
		'Adrenal' blood flow (ml./hr)		156	168	120	120	120	139
343 (male)	15·3	Secretion rates (μg/g adrenal/hr)	Aldosterone	18·8	12·7	7·2	16·1	—	22·5
			Cortisol	908·0	750·0	127·0	285·0	773·0	1448·0
			Corticosterone	608·0	300·0	90·0	135·0	593·0	847·0
		Mean blood pressure (mm Hg)		152	148	150	150	150	140
		'Adrenal' blood flow (ml./hr)		187	153	153	163	156	168

were not affected by the same chemicals. In the present experiments a mean recovery of 35·1 % with a standard deviation of ±4·2 % was obtained. All figures for secretion rates given in the Tables are corrected for 100 % recovery.

The figures obtained for cortisol and corticosterone have not been corrected for losses. The method for their estimation is less involved and recoveries average 80 % (Holzbauer & Vogt, 1961).

RESULTS

The results are summarized in Tables 1 and 2 and Figs. 1 and 2.

Secretion rate of ACTH during stress. Before hypophysectomy, the mean secretion rate of glucocorticoids (sum of cortisol and corticosterone) in eleven dogs was found to be 1814 μg/g adrenal/hr and the range 1087 to

TABLE 2. Adrenal steroid secretion, mean blood pressure and adrenal blood flow during stress, after hypophysectomy and during infusions of ACTH. Means ± standard errors calculated from results obtained in experiments on eleven dogs, including the results given in Table 1.

n = number of observations in each case. S = adrenal blood sample.

		S_1	S_2	S_3	S_4	S_5	S_6
			started 60 min after	started 90–120 min after	During infusion of ACTH		
		before			0·03	0·3	3·0
		hypophysectomy			(m-u./min/kg body wt.)		
Secetion rates (μg/g adrenal/hr) ± S.E.	Aldosterone	14·5 ± 1·28 (n = 11)	8·2 ± 1·65 (n = 11)	7·3 ± 2·41 (n = 8)	12·2 ± 1·72 (n = 8)	17·9 ± 2·21 (n = 8)	23·5 ± 2·21 (n = 7)
	Cortisol	1200·0 ± 91·0 (n = 9)	187·0 ± 71·5 (n = 10)	71·0 ± 23·4 (n = 8)	864·0 ± 117·0 (n = 8)	1527·0 ± 204·1 (n = 9)	1475·0 ± 116·3 (n = 7)
	Corticosterone	614·0 ± 55·4 (n = 9)	93·0 ± 27·4 (n = 10)	54·0 ± 11·6 (n = 8)	351·0 ± 65·0 (n = 8)	682·0 ± 60·0 (n = 9)	713·0 ± 77·1 (n = 7)
Mean blood pressure (mm Hg) ± S.E.		149 ± 4·8 (n = 11)	134 ± 4·2 (n = 11)	142 ± 3·6 (n = 8)	139 ± 2·6 (n = 8)	135 ± 6·0 (n = 9)	134 ± 6·1 (n = 7)
'Adrenal' blood flow (ml./hr) ± S.E.		233 ± 27·9 (n = 11)	202 ± 33·1 (n = 11)	195 ± 41·1 (n = 8)	180 ± 33·5 (n = 8)	181 ± 25·7 (n = 9)	168 ± 29·8 (n = 7)

2483. One hour after hypophysectomy it had decreased by 85 %, and after a further hour by 93 %. An infusion of 0·03 m-u. ACTH/min/kg body wt. increased the mean secretion rate to 66 % of the prehypophysectomy value. During the subsequent infusion of 0·3 m-u. ACTH/min/kg body wt. the secretion was increased to 120 % of the prehypophysectomy value. The infusion of 3·0 m-u. ACTH/min/kg body wt. did not cause a further rise. In Fig. 1 the infusion rates of ACTH are plotted against the secretion rates of the glucocorticoids on a semi-logarithmic scale. The diagram includes a value for the glucocorticoid secretion during an infusion of 0·01 m-u. ACTH/min/kg body wt. It was obtained in a different set of

experiments on five dogs in which the infusion of ACTH was started 2 hr after hypophysectomy and 10 min later adrenal venous blood was collected for 20–30 min whilst the ACTH infusion was continued. This value suggests that a straight-line relation between the logarithm of the infusion rate of ACTH and the glucocorticoid secretion exists up to a rate of 0·3 m-u./min/kg body wt. This straight line may be used for the estimation of the natural ACTH secretion rate before hypophysectomy. The mean glucocorticoid secretion rate observed before hypophysectomy (1814μg/g

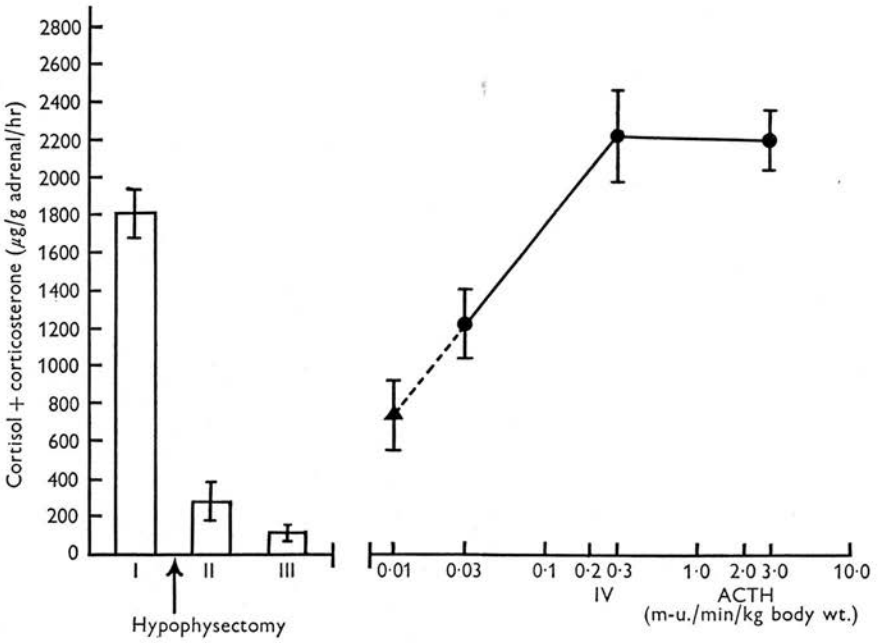

Fig. 1. Means of secretion rates of the sum of cortisol and corticosterone with standard errors. I, under operative stress; II, started 60 min after hypophysectomy; III, started 90–120 min after hypophysectomy; IV, dose–response curve between rates of glucocorticoid secretion and ACTH infusion. ▲, Point obtained in a different set of experiments (see text) from those marked with ●.

adrenal/hr) would correspond to a secretion rate of ACTH in the range of 0·03–0·3 m-u./min/kg body wt., the mean being 0·12 m-u./min/kg body wt.

Before hypophysectomy, the ratio of mean secretion rate of cortisol over mean secretion rate of corticosterone was 1·95; 2 hr afterwards, the ratio was decreased to 1·31. Infusion of 0·03 m-u. ACTH/min/kg body wt. increased it to 2·46.

Aldosterone secretion. The mean secretion rate of aldosterone before hypophysectomy was 14·5 μg/g adrenal/hr, ranging from 9·5 to 21·3 μg.

One hour after hypophysectomy it was significantly decreased to 8·16 μg (range 1·9–18 μg). There was some fall in every animal. Two hours after hypophysectomy the mean secretion rate of aldosterone was further reduced to 7·3 μg. During the infusion of 0·03 m-u. ACTH/min/kg body wt. aldosterone secretion rose in seven out of eight dogs. The mean attained was 84 % of the secretion rate before hypophysectomy. An infusion of 0·3 m-u. ACTH increased aldosterone production to 120 % and an infusion of 3·0 m-u. ACTH to 162 % of the initial value. By plotting the infusion

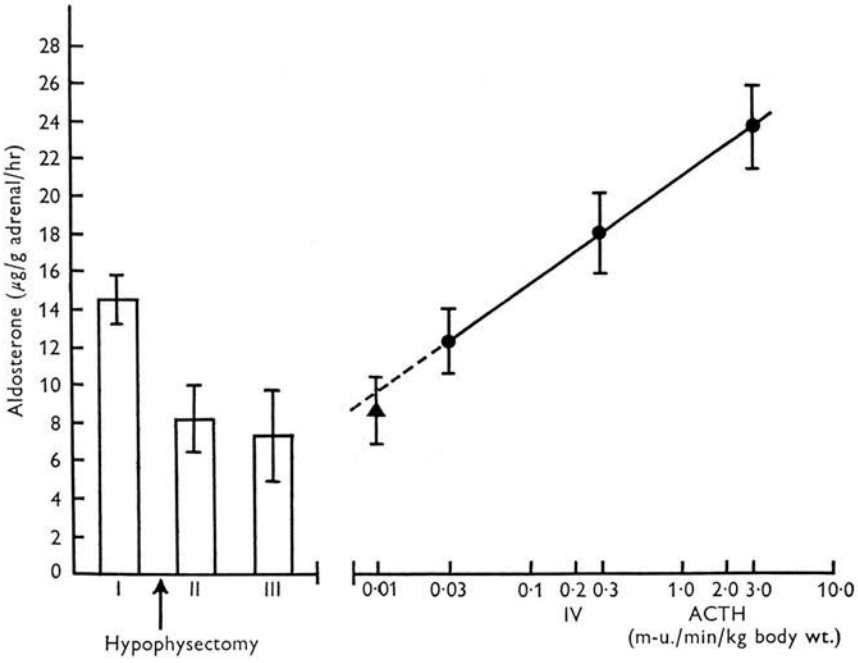

Fig. 2. Means of the secretion rates of aldosterone with standard errors. I–IV, ▲, explanation see Fig. 1.

rates of ACTH against the mean secretion rates of aldosterone on a semi-logarithmic scale (Fig. 2) a straight line dose–response curve was obtained. According to this curve an ACTH secretion rate in the range of 0·03–0·3 m-u./min/kg body wt. (the mean being 0·075 m-u./min/kg body wt.) would be necessary to obtain the mean aldosterone secretion rate observed before hypophysectomy. This estimate is of the same order as that determined from the glucocorticoid responses.

The mean blood pressure before hypophysectomy was 149 mm Hg ($\pm$ 4·8 S.E.). One hour after hypophysectomy it was significantly decreased to 134 mm Hg ($\pm$ 4·2) but after another hour it was back to the initial

value ($142 \pm 3{\cdot}6$). During the three infusions of ACTH there was a small but steady drop from $139 \pm 2{\cdot}6$ to $134 \pm 6{\cdot}1$. The mean 'adrenal' blood flow before hypophysectomy was 233 ± 28 ml./hr. After hypophysectomy it fell to 202 ± 33 in 1 hr and to 195 ± 41 in 2 hr. The ACTH infusions had no significant influence on the adrenal blood flow.

DISCUSSION

A direct assessment of the secretion rate of ACTH *in vivo* is not practicable. Therefore an indirect method has been devised for the dog, in which the animal's own adrenal was used as an indicator. Glucocorticoid secretion was measured under conditions of stress. The pituitary gland was then removed and corticoid secretion in response to ACTH infusions of different concentrations determined. By comparing the two sets of figures it was found that the mean secretion rate of ACTH during operative stress under chloralose anaesthesia was about 0·12 m-u./min/kg body wt.

Results obtained in this way are only valid if ACTH is the sole important factor which controls the secretion rate of cortisol and corticosterone. Several other polypeptides have recently been reported to affect steroid secretion in the hypophysectomized animal. One which may be of consequence in the present work is angiotensin. Davis, Ayers & Carpenter (1961) have shown that the already very low secretion rate of corticosterone is further decreased by nephrectomy in hypophysectomized, Na^+-depleted dogs. A detailed study of the response of adrenocortical secretion to infusions of renin and angiotensin was recently published by Slater, Barbour, Henderson, Casper & Bartter (1963). In sodium-loaded, hypophysectomized, nephrectomized dogs infusion of angiotensin II, 3·0 μg/min, caused a rise in the secretion rate of cortisol plus corticosterone by about 100 %. However, in absolute terms, this increase is very small indeed; the levels reached are still within the range of the secretion rates observed 2 hr after hypophysectomy in the present work on dogs which were sodium loaded and not nephrectomized. The effective doses used by Slater *et al.* caused a large rise in blood pressure and were probably far above levels occurring naturally. Thus it is unlikely that under the conditions of the present experiments renin release would have effects on the secretion rates of glucocorticoids which would vitiate the calculations carried out in the preceding paragraphs.

Another factor which could interfere with the assay are changes in the plasma concentrations of Na^+ and K^+ in the course of the experiment. Gann, Cruz, Casper & Bartter (1962) have investigated the effect of intravenous infusions of potassium chloride on the secretion rate of aldosterone and 17-hydroxy-corticosteroids (17-OHCS) in the stressed dog with intact pituitary. Increases in the plasma potassium from an initial

level of between 3 and 3·8 to concentrations up to 10 m-equiv/l. did not have any consistent effect on the secretion of 17-OHCS. Davis, Urquhart & Higgins (1963) infused concentrated solutions of potassium chloride and potassium sulphate into hypophysectomized dogs. They found that increases in the plasma potassium concentration similar to those obtained by Gann *et al.* caused significant rises in the secretion rate of corticosterone, but these changes were quite small compared to the large increase caused by ACTH. In a large series of experiments in which adrenal vein cannulation in the chloralosed dog was carried out (M. Holzbauer & M. Vogt, to be published), plasma concentrations of Na^+ and K^+ varied so little that their influence on secretion of cortisol and corticosterone can be neglected.

Before hypophysectomy, secretion rate of cortisol and corticosterone was submaximal in all dogs except no. 334 (Table 1). This would indicate either that the stress was not maximal, or that the limit of endogenous ACTH production by the pituitary had been reached.

Two hours after hypophysectomy the mean aldosterone secretion was decreased to 50 % whilst the glucocorticoid secretion was reduced to 7 % of its initial value. An infusion of 0·03 m-u. ACTH/min/kg body wt. increased the mean secretion of both aldosterone and the glucocorticoids, but neither reached the secretion rate observed during stress. An infusion of 0·3 m-u. ACTH/min/kg body wt. increased the mean glucocorticoid and the mean aldosterone secretion to 120 % of the stress value. However, when the infusion rate of ACTH was 3 m-u., the mean aldosterone secretion rose further to 162 % whereas the mean glucocorticoid production remained stationary. This indicates that in a dog with intact pituitary an increase in aldosterone secretion due to ACTH release could occur which is not accompanied by a rise in glucocorticoid secretion. This possibility had previously been discussed by Mulrow & Ganong (1961). However, it is not known whether the pituitary gland is capable of releasing these large amounts of ACTH for any length of time.

There is little doubt that ACTH is not the only, and not even the most important, factor in the control of aldosterone secretion. It is therefore to be expected that hypophysectomy or ACTH infusion will only affect aldosterone secretion *pari passu* with glucocorticoid secretion when stronger stimuli of aldosterone secretion, such as lack of sodium in the tissues, or high angiotensin concentration in the blood, are not present. This explains apparent discrepancies in the literature on the effect of hypophysectomy on secretion of aldosterone. A fall in aldosterone secretion after hypophysectomy was observed in stressed dogs by Farrell, Rauschkolb & Royce (1955), Ganong, Lieberman, Daily, Yuen, Mulrow, Luetscher & Bailey (1959), Davis, Bahn, Yankopoulos, Kliman & Peterson (1959) and others. All these authors found that ACTH increased aldosterone secretion, but to

very variable degrees. Slater *et al.* (1963), on the other hand, studied the effect of hypophysectomy on unstressed, Na^+-depleted dogs. In these animals there was no decrease in the secretion rate of aldosterone 18 hr later and little response to ACTH.

In the present experiments the extent of the fall in aldosterone secretion after hypophysectomy varied greatly although all controllable parameters were kept identical. The extremes are represented by dogs 340 and 341 (Table 1). In dog 340 aldosterone secretion was reduced by 97% 2 hr after hypophysectomy. In dog 341 it remained nearly unchanged, although, or perhaps because, the initial secretion rate was higher. An absence of aldosterone stimulating substances other than ACTH in dog 340 and an excess of them in dog 341 would explain the difference.

Differences in the effects can also be caused by the differences in the ACTH preparations. Thus Farrell, Fleming, Rauschkolb, Yatsu, McCally & Anderson (1958) found, in the decerebrated dog, that β- and δ_1- corticotrophin were equally potent in stimulating cortisol secretion but that δ_1-corticotrophin was several times more active than β-corticotrophin in stimulating aldosterone secretion. In the present work a crude preparation of ACTH was used because it was felt that it would resemble the natural secretion of the pituitary more closely than highly purified preparations.

There was a linear relation between aldosterone secretion and the logarithm of the dose of ACTH infused into the hypophysectomized dog. When the amount of ACTH corresponding to the aldosterone secretion observed before hypophysectomy was read from this line the value of ACTH was similar to the estimate of endogenous secretion of ACTH during stress calculated from glucocorticoid secretion. This may indicate that the factors other than ACTH which control aldosterone secretion remained constant in the course of the experiment. They would maintain the background secretion on which the effect of ACTH would be superimposed.

The dose–response curve for aldosterone is not as steep as that for the glucocorticoids. In order to double an initial secretion rate of 10 μg aldosterone/g adrenal/hr the amount of circulating ACTH would have to increase by a factor of 37, whereas only a 10-fold rise would be required to double a glucocorticoid secretion of 1000 μg/g adrenal/hr.

SUMMARY

1. The secretion rate of ACTH during operative stress under chloralose anaesthesia in Na^+-loaded dogs was determined by an indirect method which used glucocorticoid secretion by the dog's own adrenal as an index of circulating ACTH. The rate of release of ACTH was found to lie between 0·03 and 0·3 m-u. ACTH/min/kg body wt.

2. Under the same conditions the mean secretion rate of aldosterone

was 14·5 μg/g adrenal/hr (S.E. $\pm$ 1·28). It was decreased by 50 % 2 hr after hypophysectomy. On intravenous infusions of ACTH at rates of 0·03, 0·3 and 3·0 m-u./min/kg body wt. secretion increased linearly when plotted against the log dose of ACTH.

3. The mean secretion rate of cortisol plus corticosterone before hypophysectomy was 1814 μg/g adrenal/hr (S.E. $\pm$ 135·5). It was decreased by 93 % 2 hr after hypophysectomy. The secretion was stimulated by infusions up to 0·3 m-u. ACTH/min/kg body wt. but no further increase was achieved by ten times that dose.

My thanks are due to Dr J. B. Dekanski and Dr R. P. Edkins (Organon Labs.) for samples of ACTH. The sample of tritiated aldosterone was a gift to Dr M. Vogt by the Endocrinology Study Section, the National Institutes of Health, Bethesda, through the courtesy of Dr M. M. Graff. I am grateful to Dr M. Vogt, F.R.S., for valuable discussions and the estimation of the pressor activity of the ACTH and to Miss Helen Newport for excellent technical assistance.

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Observations on slow, rhythmic blood-pressure waves (Mayer waves) in the dog

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Among conditions which may elicit rhythmic blood-pressure waves which are independent of the respiratory rate (Mayer, 1876) are haemorrhage and asphyxia. Though chemoreceptor (Andersson, Kenney & Neil, 1950) and baroreceptor (Armstrong & Irby, 1962) activity are known to be involved in the production of the waves, the conditions determining their appearance are not well understood. In the course of a study of the effect of haemorrhage on the rate of secretion of aldosterone in the dog we had occasion to make incidental observations on the size and occurrence of Mayer waves.

The animals were anaesthetized with chloralose, heparinized, an adrenal vein was cannulated for collection of blood and the femoral blood pressure recorded with a mercury manometer for prolonged periods of time before and after withdrawal of blood (up to 25 ml./kg body wt.). A number of additional operative procedures was carried out on some of the dogs and the results, summarized in Table 1, suggest the following conclusions:

(1) In all groups, a certain number of dogs failed to develop Mayer waves after haemorrhage; only in some of the dogs was this failure accompanied by, and probably due to, a less severe fall in blood pressure after bleeding.

(2) In a few dogs of groups 1, 3 and 8, all of which had been subjected to surgical procedures as recently as half an hour previously, Mayer waves were present before haemorrhage, yet these dogs did not necessarily have a low initial blood pressure.

(3) If the blood pressure after bleeding fell below 50 mm Hg, the Mayer waves usually disappeared.

(4) Denervation of the carotid sinuses and of the origin of the thyroid arteries diminished neither frequency of occurrence nor amplitude of Mayer waves.

(5) Section of the splanchnic nerves or evisceration reduced the amplitude but not the frequency of occurrence of Mayer waves.

(6) The smallest amplitudes of Mayer waves were seen 2–4 hr after hypophysectomy (in spite of the infusion of ACTH). Impairment of vasomotor tone in this group was indicated by low initial blood pressures.

It was surprising to find that anoxia produced by breathing gas mixtures with a low oxygen content or by replacing the dog's blood with plasma till the haematocrit was halved, did not elicit Mayer waves, but that angiotensin infusions elicited them in hypophysectomized-nephrectomized dogs.

TABLE 1. Occurrence, mean amplitude (mm Hg) and mean frequency (per min) of Mayer waves after abdominal surgery. Observations were made on dogs under chloralose anaesthesia and started about 30 min after dissection of an adrenal vein, except for groups 2 and 4 in which a rest period of 2½ hr was allowed

Group	Surgical procedure	No. of dogs		Haemorrhage Before	Haemorrhage After	Haemorrhage Before	Haemorrhage After	Haemorrhage Before	Haemorrhage After	Haemorrhage Before	Haemorrhage After
1	No additional operation	16	No. of dogs	2		11		1		2	
			Occurrence of waves	—	—	—	+	+	—	+	+
			M.b.p., mm Hg	145	93	142	81	100	60	155	88
			Amplitude (frequency)	—	—	—	29 (3·1)	40 (4·7)	—	20 (4·0)	43 (2·8)
2	No additional operation, 2½ hr rest	7	No. of dogs	1		6		—		—	
			Occurrence of waves	—	—	—	+	—	—	—	—
			M.b.p., mm Hg	165	120	136	103	—	—	—	—
			Amplitude (frequency)	—	—	—	33 (4·7)	—	—	—	—
3	Carotid sinus denervation, 7–14 days previously	5	No. of dogs	2		3		—		—	
			Occurrence of waves	—	—	—	+	—	—	—	—
			M.b.p., mm Hg	190	123	182	100	—	—	—	—
			Amplitude (frequency)	—	—	—	33 (3·1)	—	—	—	—
4	R. nephrectomy, 7–14 days, L. nephrectomy 30 min. before dissection of adrenal vein; 2½ hr rest	10	No. of dogs	2		8		—		—	
			Occurrence of waves	—	—	—	+	—	—	—	—
			M.b.p., mm Hg	173	100	171	110	—	—	—	—
			Amplitude (frequency)	—	—	—	28 (4·9)	—	—	—	—
5	Acute splanchnotomy	7	No. of dogs	2		2		1		2	
			Occurrence of waves	—	—	—	+	+	—	+	+
			M.b.p., mm Hg	123	75	118	58	85	35	115	60
			Amplitude (frequency)	—	—	—	11 (2·7)	30 (2·8)	—	22·5 (3·7)	20 (2·4)
6	Splanchnotomy, 7–14 days previously	8	No. of dogs	2		6		—		—	
			Occurrence of waves	—	—	—	+	—	—	—	—
			M.b.p., mm Hg	160	110	143	88	—	—	—	—
			Amplitude (frequency)	—	—	—	15 (3·2)	—	—	—	—
7	Evisceration	9	No. of dogs	4		3		—		2	
			Occurrence of waves	—	—	—	+	—	—	+	+
			M.b.p., mm Hg	161	86	138	75	—	—	140	65
			Amplitude (frequency)	—	—	—	13 (2·2)	—	—	14 (3·5)	21 (1·9)
8	2 hr after hypophysectomy, infusion of ACTH	14	No. of dogs	6		3		1		4	
			Occurrence of waves	—	—	—	+	+	—	+	+
			M.b.p., mm Hg	121	75	148	77	115	65	124	95
			Amplitude (frequency)	—	—	—	10 (3·2)	6 (1·5)	—	16 (4·0)	15 (3·7)

The appearance of Mayer waves is indicated by a +.

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Gas chromatography of androgens, progesterone and progesterone derivatives in adrenal venous blood of pigs and dogs

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The secretion of steroids less polar than corticosterone is being studied by analysis of adrenal venous blood collected from pigs and dogs under anaesthesia. This group of steroids includes the adrenal androgens and precursors of the major corticosteroids. Qualitative and quantitative gas–liquid chromatography using flame-ionization detection after preliminary separation by paper chromatography in different solvent systems is employed. This enables the detection of a wide range of the above steroids. Further identification includes the analysis of reaction products after acetylation or after enzymic conversion with 20β-hydroxysteroid dehydrogenase and the examination of ultraviolet absorption and spectra of sulphuric acid chromogens.

In adrenal blood of both species significant amounts of four compounds less polar than corticosterone were observed in addition to cholesterol. The first two had properties similar to 11βOH-progesterone and 11βOH-androstenedione, respectively, the third to pregnenolone and the fourth to progesterone. In addition pig adrenal blood contained small quantities of steroids which resemble androstenedione, 16αOH- and 17αOH-progesterone in their chromatographic behaviour.

A quantitative method for the estimation of nanogram amounts of steroids by gas chromatography was developed. After adding a known amount of a reference steroid, the amount of the unknown steroid was calculated from the ratio of their peak heights.

After hypophysectomy there is a severe decrease in the secretion rate of the main steroids under investigation.

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ADRENAL SECRETION RATES OF C-19 AND C-21 STEROIDS BEFORE AND AFTER HYPOPHYSECTOMY IN THE PIG AND THE DOG

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SUMMARY

(1) The adrenal secretion of androgens and of some intermediates of corticosteroid biosynthesis have been studied in immature pigs and adult dogs under anaesthesia.

(2) Qualitative and quantitative analyses of the extracts of adrenal venous blood were performed by a combination of paper and gas chromatographic techniques.

(3) In both species 11βOH-androstenedione, pregnenolone, progesterone and 11βOH-progesterone were secreted under stress conditions at a mean rate of approximately 20 μg./adrenal/hr.; this was somewhat more than aldosterone secretion and about one-fiftieth of the glucocorticoid secretion.

(4) Within 2 hr. of hypophysectomy the secretion of all four compounds was decreased by more than 95%.

INTRODUCTION

The presence of steroids with androgenic activity in adrenal venous blood and in perfusates of the gland has been described for a number of species. Their excessive secretion caused by aberrations in adrenal steroid metabolism and by adrenal tumours in human patients has been extensively studied (for reviews see Dorfman & Shipley, 1956; Short, 1960; Howard & Migeon, 1962). Little is known, however, about the physiological aspects of adrenal androgen secretion, e.g. whether these substances are secreted regularly by the adrenal gland, what their secretion rates are and which factors control their secretion.

This paper attempts to answer some of these questions. Adrenal venous blood was collected under anaesthesia from immature pigs and adult dogs. Gas chromatographic methods were used for the detection and determination of androgens in blood extracts. After it had been established which compounds were secreted in significant amounts their rates of secretion were determined under conditions of operative stress. The effect of hypophysectomy on these secretion rates was then studied in dogs.

During the course of this work measurable amounts of some steroids known to be precursors of corticosteroids were found. The secretion rates of these compounds

were also studied as were, in the pig, those of glucocorticoids and aldosterone for which there is no information.

A short report on these experiments has already been published (Heap & Holzbauer, 1966).

METHODS

Operative procedures

Pigs

All pigs used were taken from a herd bred in the Institute which was free from enzoötic pneumonia.

Pig I (Landrace, male, 7 weeks old, 14 kg.). Anaesthesia was induced with halothane followed by chloralose (Merck, 80 mg./kg., 0·7% solution in 0·9% NaCl) injected into a femoral vein through a catheter, the tip of which was advanced towards the inferior vena cava. After chloralose had been given, approximately 20 units heparin were injected through the same catheter every ½ hr. to keep the cannula patent. Later in the experiment several doses of a 5% solution of pentobarbitone were given intravenously to maintain the required depth of anaesthesia. The trachea was cannulated in order to be able to apply artificial respiration by a Starling pump (20 rev./min., stroke 15 ml./kg. body weight). The region of the left adrenal was approached extraperitoneally by a flank incision. The gland was found on the ventral surface of the aorta, its caudal end lying near to the left renal vein. A very short adrenal vein led into the renal vein. The left kidney was removed and the junction between the renal vein and the vena cava exposed. Dissection of this region was difficult because in the young pigs used the veins were very fragile and surrounded by very much tougher connective tissues. A ligature was placed round the renal vein near its entry into the vena cava. A polythene cannula was introduced into the renal vein after tributaries coming from extra-adrenal tissues had been tied, and after heparin (500 units/kg.) had been injected. The renal vein was tied at its junction with the vena cava and adrenal blood collected during four consecutive periods of 45 min. each. The blood pressure was recorded from the abdominal aorta by a polythene cannula inserted through a femoral artery. It was maintained by an infusion of Dextraven (dextran, 6 g./100 ml., in glucose solution, 5 g./100 ml., salt free, Benger Laboratories Ltd.) given intravenously during the last two collection periods.

Pig II (Landrace, female, 9 weeks old, 15 kg.). In this animal anaesthesia was maintained with halothane throughout the experiment. The left adrenal was again approached extraperitoneally from a flank incision. During the dissection the adrenal vein was torn at its emergence. After stopping all other bleeding points around the vein adrenal blood was collected for half an hour with a syringe as it issued from the gland.

In one pig the left adrenal was not drained by the renal vein; in another the gland was sitting directly on the junction between vena cava and renal vein. It was not possible to collect blood from these two animals by the technique described above, and another method was therefore adopted for the subsequent animals.

Pig III (Large White, female, 13 weeks old, 17·7 kg.). Anaesthesia was the same as in pig I, but additional pentobarbitone was not required. A mid-line incision was

made and the viscera and both kidneys were removed. The region of the inferior vena cava adjoining both adrenal glands was then freed from the surrounding tissues and any accessible tributaries not coming from the adrenals were tied. One ligature was tied round the vena cava at its entry into the liver and another about 2 cm. caudal to the mouth of the left adrenal renal vein. A cannula was inserted into the ligated section of the vena cava about 1 cm. cranially to the distal ligature. Adrenal venous blood draining from both glands into this segment was collected for 20 min.

Pig IV (Large White, female, 9 weeks old, 12·6 kg.). Anaesthesia and dissection were as in pig III. Eight blood samples were collected during 10 min. periods. During the second period a shunt flow was established between the caudal and cranial sections of the inferior vena cava left after cannulating the middle section of the vein. Dextraven infusions were started during the third collection period and continued until the pig died suddenly at the end of the eighth collection period.

Dogs

Adult mongrel dogs of both sexes were used. Anaesthesia was induced with ether; chloralose (Merck, 70 mg./kg. body weight as 0·7% solution in 0·9% NaCl) was then infused into a femoral vein. A tracheal cannula was inserted. Cannulation of the left adrenal vein, hypophysectomy and preparation of the donor blood were carried out as described previously (Holzbauer, 1964). Adrenal venous blood was collected for periods of 25–40 min., depending on the size of the adrenal gland.

In dogs 1, 2 and 3 the pituitary was left intact and only one adrenal blood sample was collected. In dogs 4, 5 and 6 the pituitary was removed 2 hr. before collection of the first blood sample. Altogether four samples were collected from each dog at regular intervals during 3½ hr. Since these three dogs were also used to investigate another aspect of steroid secretion, their kidneys were removed and the donor blood was obtained from nephrectomized animals. In dogs 7 and 8 the first sample was collected before, and the second sample 2 hr. after, hypophysectomy. By this time glucocorticoid secretion has usually fallen by about 90% (Holzbauer, 1964). In dog 9 the same procedure was carried out, but hypophysectomy was later found to have been incomplete. At the end of each experiment the skull was opened and the base of the brain searched for pituitary remnants.

Chemical procedures

Chemicals

All chemicals were of A.R. grade and when necessary were purified as described previously (Holzbauer & Vogt, 1966). Aldehyde-free ethanol was prepared according to the U.S. Pharmacopoeia (15th edn.) and distilled a second time. Antimony trichloride was stored over phosphorus pentoxide and the chloroform was washed three times with distilled water, dried over sodium sulphate and distilled. The silica gel used for thin-layer chromatography was Mallinckrodt SilicAR-7GF with fluorescent indicator. The enzyme used for the reduction of progesterone was 20β-hydroxysteroid dehydrogenase (Boehringer & Sons). All steroid standards were obtained in crystalline form from the M.R.C. Steroid Reference Collection. Radioactive steroids, [7-^{3}H]aldosterone (sp.act. 13 μc/μg.) and [4-^{14}C]progesterone (sp.act. 69 μc/mg.)

were added to correct for losses during processing. The purity of radioactive steroids was checked by paper chromatography.

Preparation of blood extracts

Whole blood was extracted and the extracts purified as described previously (Holzbauer, 1964). For the determination of cortisol and corticosterone one-twentieth of the extract was treated as described by Vogt (1955).

Paper chromatography

The extracts were applied to 4 cm. strips of washed Whatman no. 2 paper (Holzbauer & Vogt, 1961) and subjected to chromatography in Bush's system B_3 (Bush, 1952) for 3 hr. Compounds less polar than androst-4-ene-3,11,17-trione were rechromatographed in Bush's system A for 4 hr., and compounds of equal polarity for 16–20 hr. Compounds of higher polarity were run a second time in system B_3 for 12 hr.

For aldosterone determinations the origin of the first B_3 chromatogram was eluted and rechromatographed in the E_2B system (Eberlein & Bongiovanni, 1955). The further procedures are described elsewhere (Holzbauer & Vogt, 1966).

Control spots of Δ^4-3-ketosteroids were located on paper chromatograms by their absorption of ultraviolet light, and of 17-ketosteroids by a modified Zimmermann reaction (Schindler & Reichstein, 1951). The antimony trichloride reaction described by Neher & Wettstein (1951) was also used.

Thin-layer chromatography

The solvent system was methanol:benzene (1:9, v/v). Steroids were located by their absorption of ultraviolet light and by their reactions with phosphomolybdic acid in acetone (10%, w/v) or with anisaldehyde as used by Lisboa (1965).

Gas–liquid chromatography (GLC)

Areas on the paper chromatograms of blood extracts which corresponded to the position of various markers were eluted. The eluates were analysed on a Model 400, F and M Scientific Corporation gas chromatograph incorporating a flame ionization detector and a Honeywell recorder.

The column packings were prepared by the method of Wotiz & Chattoraj (1964) and consisted of 3·8% SE-30 on silanized Diatoport S (60–80 mesh). After packing, the glass U-shaped columns (6 ft. by 4 mm. i.d.) were conditioned at 300° for 12 hr. with a carrier gas (Argon) flow rate of 10 ml./min. These columns were operated under the following conditions: column temperature, 220–230°; detector temperature 10–20° higher; flash heater, 300°; gas flows: Argon (carrier) 50 ml./min. at 2·8 kg./cm.^{2}; hydrogen, 40 ml./min.; air, 300 ml./min.

Peak heights or peak areas (peak height × width at half peak height) were used for quantitation. Relative retention times are quoted with respect to cholestane and they were calculated from the distance between the first deflexion of the pen recorder, which occurred immediately after sample injection, and the apex of each peak.

Other procedures

Acetylation. This was carried out by dissolving the dry sample in 0·04 ml. pyridine and 0·16 ml. acetic anhydride and incubating for 2 hr. in a water bath at 60°. This is sufficient to produce the diacetate of aldosterone.

Estimation of progesterone. In a number of samples progesterone was also estimated by a fluorescence method after its enzymic conversion into 20β-hydroxypregn-4-en-3-one. Full details of the method and of the measurement of radioactivity are published elsewhere (Heap, 1964).

Spectra in sulphuric acid. These were prepared by dissolving the steroids in 78% sulphuric acid. The absorption spectra were plotted in a recording Unicam spectrophotometer over a range from 200 to 600 mμ. Blank values were deducted simultaneously.

Quantitative estimation by gas chromatography

A quantitative method to estimate adrenocortical steroids by gas chromatography based on the straight-line relation between detector response and the amounts of injected steroid was used. Figure 1 shows the values of peak heights (Fig. 1A) and of peak areas (Fig. 1B) produced by different amounts of seven steroids. These lines, calculated for each steroid from a number of observations on different amounts, showed that the sensitivity of this method was different for various compounds. The limits of detection lay between 2 and 10 ng. Coefficients of variation of peak heights were about 15%; in some instances they were as low as 5% and in others as high as 35%. These large errors, principally due to the technical problem of applying small quantities of volatile solvents to the column, made the simple relation between steroid quantity and peak size unsuitable for quantitative purposes.

However, by injecting a known amount of a reference steroid simultaneously with the sample, the injection of a precise volume was no longer necessary for an accurate estimate of the unknown. Similar techniques have been used previously for the quantitative estimation of steroids by gas chromatography (e.g. Vandenheuvel, Hinderks, Nixon & Layng, 1965; Wisniewski & Umbreit, 1965).

Calibration lines were prepared with mixtures of pure steroids in absolute ethanol in which the ratio between the concentrations of the reference steroid and of the steroid to be assayed varied as shown in Figs. 2A and B. Reference compounds were selected so that their relative retention time (RRT) was significantly different from that of the 'unknown' steroid and from other endogenous compounds. All pipetting was carried out with automatic micropipettes, and the mixtures were kept at $-17°$ for several weeks. According to the principle of the method small solvent losses could be neglected. When peak height ratios were calculated the coefficient of variation of a number of observations at a given ratio was 2–5% provided the ratio was within the range of 0·05 to 4·5.

To estimate the amount of a steroid present in a biological sample the dried eluate from a paper chromatogram was taken up in 50 or 100 μl. ethanol. Volumes of 1 μl. were applied to the gas chromatograph with or without cholestane. The remainder of the sample was taken to dryness and dissolved in an accurate amount (25–200 μl.) of ethanol which contained the appropriate 'reference steroid' (100 ng./μl.).

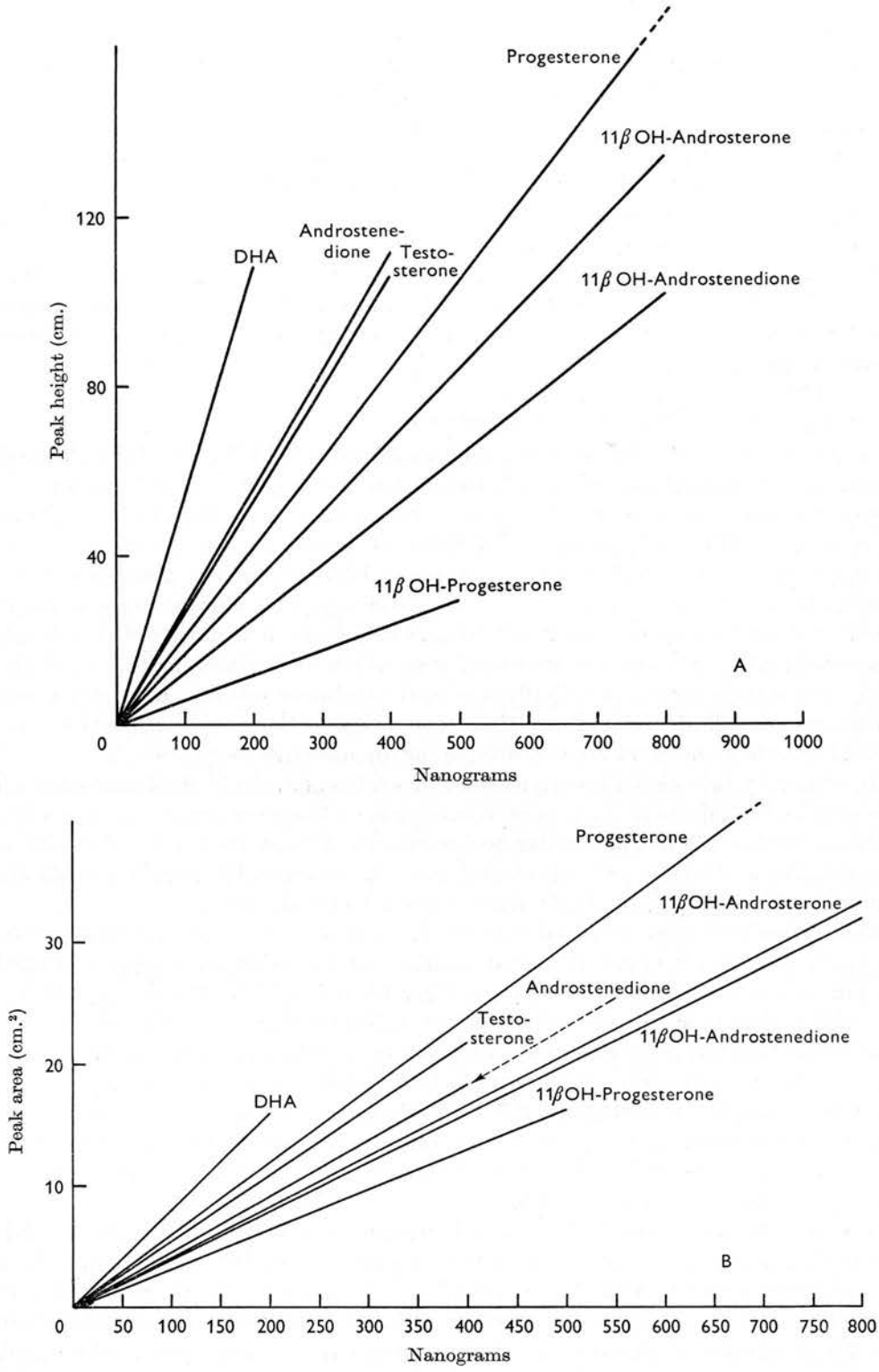

Fig. 1. Linear relationship between amounts of steroid applied to the gas chromatograph and observed peak heights (A) or peak areas (B) as calculated for seven steroids.

After GLC the peak heights of the steroid to be estimated (X) and of the reference steroid (A) were measured and the ratio $X:A$ calculated. For each calculated ratio of $X:A$ the corresponding concentration ratio in the solution (conc. X:conc. A) was read off from a calibration curve (Fig. 2) and then multiplied by the amount of the reference steroid added, to obtain the amount of the steroid contained in the paper eluate.

Standard lines were always constructed on the day of the estimations by injecting standard mixtures between unknown samples. Although calibration curves were reproducible, small changes in the slopes were sometimes observed.

The losses involved in processing blood samples, as assessed by adding 5–10 μg. of steroids to 100 ml. of arterial blood, ranged from 20 to 50% for different steroids.

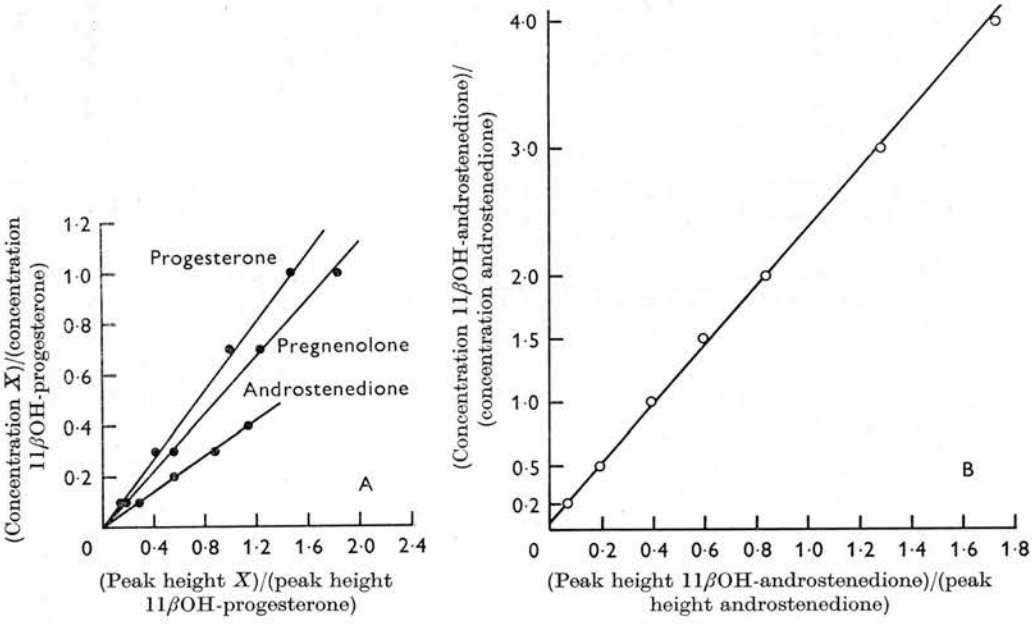

Fig. 2. Straight-line relationships for the quantitative estimation of progesterone, pregnenolone, androstenedione (A) and of 11βOH-androstenedione (B) by gas chromatography. Calibration lines obtained by plotting: (peak height X)/(peak height of reference steroid) against (concentration of X)/(concentration of reference steroid), where X was the steroid to be assayed and where the reference steroid was 11βOH-progesterone (A) or androstenedione (B). All points are single observations. Not more than 500 ng. of steroid was applied.

RESULTS

Chromatographic data

Table 1 lists the trivial and chemical names of steroids referred to in this paper. It shows the R_F values in the B_3 system and the relative retention times for 32 steroids which may occur in adrenal venous blood. The R_F values of these compounds in the B_3 system were either greater than or similar to that of corticosterone. Only the first 27 gave discrete peaks when subjected to gas chromatography in amounts of 2–10 ng., with the exception of deoxycorticosterone which gave inconsistent results.

Table 1. *Some characteristics of 32 steroids*

No.	Systematic name	Trivial name	R_F values*	Relative retention time†		Antimony trichloride reaction‡	
				Before acetylation	After acetylation	Daylight	Ultraviolet light
1	3α-Hydroxy-5α-androstan-17-one	Androsterone	0·82	0·37	n.d.	Bluish pink + + +	Pink + + + + +
2	3α,11β-Dihydroxy-5α-androstan-17-one	11βOH-Androsterone	0·23	0·64	n.d.	Mauve + + +	Pink orange + + +
3	3β-Hydroxy-5α-androstan-17-one	Epiandrosterone	0·86	0·38	n.d.	Bluish pink + +	Pink orange + +
4	3β,11β-Dihydroxy-5α-androstan-17-one	11βOH-Epiandrosterone	0·15	0·65	n.d.	Bluish pink (+)	Brownish (+)
5	3β-Hydroxyandrost-5-en-17-one	Dehydroepiandrosterone	0·75	0·39	0·57	Red + + + + +	Rust red + + + + +
6	3α-Hydroxy-5β-androstan-17-one	Aetiocholanolone	0·78	0·34	n.d.	Bluish pink (+)	Pink orange + + + +
7	3α,11β-Dihydroxy-5β-androstan-17-one	11βOH-Aetiocholanolone	0·26	0·49	n.d.	Grey purple + + +	Brown (+)
8	3α-Hydroxy-5β-androstane-11,17-dione	11Keto-aetiocholanolone	0·39	0·43	n.d.	Grey (+)	Grey (+)
9	Androst-4-ene-3,17-dione	Androstenedione	0·83	0·50	0·48	Pink +	Pale pink + +
10	11β-Hydroxyandrost-4-ene-3,17-dione	11βOH-Androstenedione	0·26	0·84	0·84	Mauve + + +	Orange + + +
11	Androst-4-ene-3,11,17-trione	Adrenosterone	0·49	0·59	0·58	Pale brown (+)	Pale pink (+)
12	17β-Hydroxyandrost-4-en-3-one	Testosterone	0·56	0·53	n.d.	Pink + + + + +	Orange + + + + +
13	17α-Hydroxyandrost-4-en-3-one	Epitestosterone	0·66	0·53	n.d.	Mauve + + + + +	Orange + + + + +
14	3-Hydroxyoestra-1,3,5(10)-trien-17-one	Oestrone	0·57	0·47	n.d.	Yellow +	Lime green +
15	Oestra-1,3,5(10)-triene-3,17β-diol	Oestradiol-17β	0·33	0·51	n.d.	Yellow +	Yellow + + +
16	Oestra-1,3,5(10)-triene-3,17α-diol	Oestradiol-17α	0·82	0·68	n.d.	Yellow + + + + +	Yellow green + + + + +
17	Pregn-4-ene-3,20-dione	Progesterone	0·97	0·82	0·82	Brownish (+)	Grey (+)
18	17α-Hydroxypregn-4-ene-3,20-dione	17αOH-Progesterone	0·51	1·16	1·17	Pink + +	Pink yellow + + + + +
19	16α-Hydroxypregn-4-ene-3,20-dione	16αOH-Progesterone	0·16	0·77	n.d.	Grey (+)	Grey (+)
20	11β-Hydroxypregn-4-ene-3,20-dione	11βOH-Progesterone	0·46	1·46	1·45	Pink (+)	Grey (+)
21	Pregn-4-ene-3,11,20-trione	11Keto-progesterone	0·71	1·07	n.d.	Grey (+)	Grey (+)
22	20α-Hydroxypregn-4-en-3-one	—	0·76	0·92	1·3	Blue (+)	Grey +
23	3β-Hydroxypregn-5-en-20-one	Pregnenolone	0·86	0·63	0·92	Purple + + + +	Pink orange + + + +
24	3β,17α-Dihydroxypregn-5-en-20-one	17αOH-pregnenolone	0·0	0·86	n.d.	Orange-pink + +	Orange + +
25	3β-Hydroxycholest-5-ene	Cholesterol	1·00	2·0	n.d.	Purple + + + +	Grey (+)
26	21-Hydroxypregn-4-ene-3,20-dione	Deoxycorticosterone	0·63	1·52	2·22	Pink + +	Pink (+)
27	11β,21-Dihydroxypregn-4-ene-3,20-dione	Corticosterone	0·08	1·2, 1·5, 1·85	3·8	Pink + +	Pink + + + + +
28	11β,17α,21-Trihydroxypregnene-3,20-dione	Cortisol	0·02	0·87	n.d.	Pink + +	Yellow grey + + + +
29	17α,21-Dihydroxypregn-4-ene-3,11,20-trione	Cortisone	0·03	—§	n.d.	Brownish blue (+)	Yellow pink + +
30	17α,21-Dihydroxypregn-4-ene-3,20-dione	17OH-11-Deoxycorticosterone	0·10	—§	n.d.	Purple + + + + +	Orange + + + + +
31	21-Hydroxypregn-4-ene-3,11,20-trione	11-Dehydrocorticosterone	0·15	—§	n.d.	Brownish blue +	Yellow pink + +
32	11β,21-Dihydroxy-3,20-dioxopregn-4-en-18-al	Aldosterone	0·0	—§	n.d.	Grey (+)	Grey (+)

* R_F values in Bush system B_3 for 3 hr.

† Relative retention time measured by gas chromatography on 3·8 % SE-30 column. For other conditions see text, p. 162.

‡ Antimony trichloride reaction (see text, p. 162): + + + + +, very intense; (+) hardly visible.

§ Not recovered in gas–liquid chromatography.

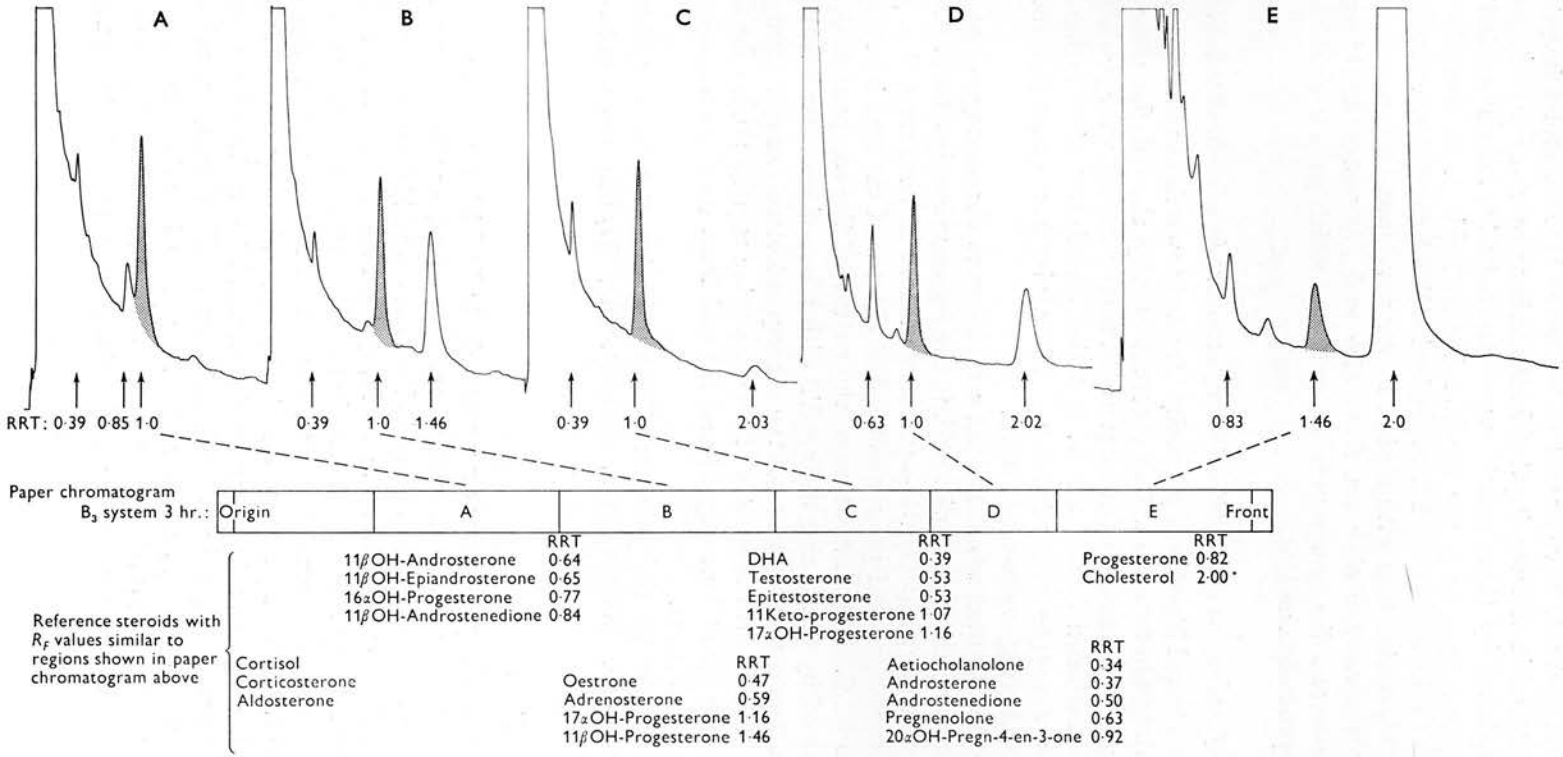

Fig. 3. Analysis of adrenal blood extract of dog no. 2. Lower diagram: paper chromatogram of blood extract (system B_3, 3 hr.) with positions of various authentic steroids and their RRT values measured by gas–liquid chromatography (GLC). Upper diagram: GLC tracings of eluates of regions A–E on the paper chromatogram, with RRT values of the major peaks (relative to cholestane). Tracings A–D range 1, attenuation 8; tracing E range 1, attenuation 16. Chart speed 0·625 cm./min. Shaded peaks in tracings A–D: cholestane, added for RRT calculation; E: 11βOH-progesterone, reference steroid added for quantitation (RRT = 1·46). For conditions of gas chromatography see text, p. 162.

Most of these steroids should have been detected in the blood samples investigated if they had been present in an unconjugated form and in amounts of more than 0·2 μg.

The colours produced by these steroids when treated with anhydrous antimony trichloride on chromatography paper are included in Table 1. In some instances a specific colour was developed, an intense red with DHA (5), intense mauve with epitestosterone (13), contrasting with a pink colour developed by testosterone (12) or a lime-yellow fluorescence obtained from 17α- and 17β-oestradiol (16 and 15). Among the progesterone-like compounds the only one which gave a good reaction was 17α-hydroxyprogesterone (18).

Identification of androgens and corticosteroid precursors in adrenal venous blood

The analysis of adrenal blood was primarily directed towards the identification of steroids with androgenic activities. Gas chromatography showed the presence of measurable amounts of a number of other steroids, three of which were predominant and are therefore included in this study. Detailed qualitative observations were carried out on blood samples of pig I; analysis of samples from other pigs and dogs was mainly confined to R_F values and RRT.

In pig I successive purified adrenal blood samples were chromatographed in the system B_3. Each chromatogram was divided into five regions according to the positions of the following control spots: 11βOH-androstenedione, adrenosterone, deoxycorticosterone, androstenedione and progesterone. The eluates of the first blood sample were subjected to GLC. Some indication of the nature of the compounds detected by GLC was obtained by comparing their RRT with those of pure steroids that had similar R_F values. The chromatograms of the subsequent blood samples were cut up in a similar way, eluted and the eluates of corresponding regions pooled for further tests. Adrenal venous blood from pigs II and III and from six dogs was treated in a similar manner (see Fig. 3).

Presumptive evidence for the presence of the following steroids was obtained: 11βOH-androstenedione, pregnenolone, progesterone, 11βOH-progesterone and cholesterol.

11βOH-Androstenedione

When the region of the paper chromatogram of pig I which corresponded to the position of this steroid was eluted and gas-chromatographed, a major peak occurred with an RRT of 0·84. Of the steroids listed in Table 1, only 11βOH-androstenedione had both the same RRT and R_F value in the B_3 system. The RRT was not affected by attempted acetylation of either the sample or of the pure steroid. The 11βOH-androstenedione areas of the remaining samples of blood from pig I were rechromatographed for 16 hr. in the B_3 system and showed one strongly ultraviolet light-absorbing spot in the region of 11βOH-androstenedione. The elution of this area and subsequent GLC gave a single large peak with an RRT of 0·84, an ultraviolet absorption maximum at 240 mμ, an absorption spectrum in sulphuric acid and a fluorescence spectrum in H_2SO_4 : ethanol (4 : 1) similar to those of the authentic steroid. In adjacent regions of the same paper chromatogram small peaks with the same RRT were found.

Pregnenolone

The eluate of the pregnenolone area of the paper chromatogram of blood from pig I showed a peak with an RRT of 0·63 (after acetylation, 0·92). The only compound in Table 1 with the same R_F and RRT values both before and after acetylation was pregnenolone. The pregnenolone regions of blood samples 2–4 were rechromatographed in system A for 4 hr. Only the eluate corresponding to the region of pregnenolone showed a single large peak with an RRT of 0·63. Further identification of pregnenolone in a sample of dog adrenal blood was performed by measuring its R_F value (0·53) after thin-layer chromatography (R_F of authentic pregnenolone, 0·53). The steroid was located by anisaldehyde, which produced a mauve colour, and by phosphomolybdic acid.

Progesterone

The progesterone regions of paper chromatograms of pig I showed ultraviolet absorption and their eluates exhibited single peaks with an RRT of 0·82, similar to that of authentic progesterone. Eluates were treated with the enzyme 20β-hydroxysteroid dehydrogenase and a fluorescence corresponding to 20β-hydroxypregn-4-en-3-one in ethanol:H_2SO_4 (1:2) was detected in all instances.

11βOH-Progesterone

When the eluate of the 11βOH-progesterone region of the paper chromatogram of the first blood sample of pig I was subjected to GLC a large peak was obtained with an RRT of 1·45. This was not affected by acetylation. The only steroid in Table 1 with the same properties was 11βOH-progesterone. The remaining eluates of the 11βOH-progesterone region were rechromatographed in system A for 22 hr. A strongly absorbing spot was visible in ultraviolet light which had the same R_F value as 11βOH-progesterone and gave a large peak with an RRT of 1·45. A sulphuric acid chromogen of this region had similar absorption peaks to those of the pure steroid. After eluates of the same region were enzymically converted with 20β-hydroxysteroid dehydrogenase, gas chromatography showed a large peak with an RRT of 1·45, and after acetylation an RRT of 1·99, which were the same as authentic 11β,20β-dihydroxypregn-4-en-3-one and 11β-hydroxy-20β-acetoxypregn-4-en-3-one, respectively.

Cholesterol

Eluates of the solvent front regions of paper chromatograms always produced very large peaks with an RRT of 2·0 indicating the presence of considerable amounts of cholesterol. Cholesterol was further identified in extracts of adrenal venous blood of a dog after thin-layer chromatography and spraying with phosphomolybdic acid.

In addition to the above steroids, which appear to be regular components of adrenal venous blood in pigs and dogs, some evidence was also obtained about the following steroids:

Deoxycorticosterone (*DOC*). Gas chromatography of an eluate of the region of the paper chromatogram corresponding to DOC (pig I, sample 1) produced a large peak with the RRT of pure DOC (RRT = 1·54). On rechromatography in system A for

16 hr. an ultraviolet absorbing spot was located in the DOC region; its eluate gave a large peak with RRT 1·54 and, after acetylation, 2·26.

Other compounds. In adrenal venous blood of pigs and dogs some very small peaks were detected by GLC which corresponded to the RRT of androstenedione, adrenosterone and 16αOH- and 17αOH-progesterone. A significant amount of DHA was possibly present in the first sample from pig I. The eluate of the paper chromatogram area which should have contained any DHA gave a peak with an RRT of 0·39 (after acetylation, 0·57). When eluates of the DHA regions of the remaining blood samples were pooled and rechromatographed, DHA was not found. In later experiments the presence of DHA may have been obscured by a 'paper blank' peak (RRT = 0·39; after acetylation, 0·56). In both species a number of unidentified compounds were present; some of these had a relative retention time greater than 2·0 and occurred in significant amounts.

Arterial blood

Arterial blood samples were collected from a femoral artery (100 ml.) and examined in the same way as adrenal venous blood. Eluates of all regions of the paper chromatograms gave peaks similar to those of paper blanks (RRT = 0·39 and 1·07) and in addition a small peak at 0·75. The eluates of regions which included the solvent front contained large quantities of cholesterol. From these observations it was assumed that neither adrenal androgens nor the three corticosteroid precursors studied are present in arterial blood in concentrations exceeding 0·2 μg./100 ml.

Secretion rates in pigs

All observations were made on 'stressed' pigs with intact pituitary glands. The secretion rates of 11βOH-androstenedione, pregnenolone, progesterone and 11βOH-progesterone were measured in pig III (Table 2). Those of cortisol, corticosterone and aldosterone were estimated in pig I (4 samples) and pig IV (8 samples) (Table 3).

Secretion rates in dogs

Pituitary intact

Table 4 shows observations made in six stressed dogs with intact pituitary glands. The left adrenals of these dogs secreted 11βOH-androstenedione at a mean rate of 18 μg./hr., the figures ranging from 6 to 31. The three corticosteroid precursors were secreted at rates ranging from 4 to 44 μg./hr. The mean secretion rates were: pregnenolone, 22·8; progesterone, 12·3; 11βOH-progesterone, 24·1 μg./hr.

Pituitary removed 2 hr. previously

In adrenal venous blood of hypophysectomized dogs collected for the same period of time as in intact dogs and measured under identical conditions, 11βOH-androstenedione and the three major corticosteroid precursors were hardly detectable (Table 4). In Fig. 4 an example is shown of the occurrence of 11βOH-progesterone in adrenal venous blood of a dog collected before (A) and 2 hr. after (B) hypophysectomy. In sample A there was a large peak with an RRT of 1·45, whereas only a negligible response was recorded from a similar aliquot of sample B. Similar observations were made on the other two corticosteroid precursors. The secretion of 11βOH-androstenedione was also greatly reduced by hypophysectomy and in several instances this

Table 2. *Secretion rates of 11βOH-androstenedione, pregnenolone, progesterone and 11βOH-progesterone in pig III*

('Adrenal' blood flow: 550 ml./hr., approximate weight of adrenal: 0·9 g.)

Compound	Secretion rate (μg./adrenal/hr.)
11βOH-Androstenedione	14
Pregnenolone	10
Progesterone	13
11βOH-Progesterone	19

Table 3. *Secretion of cortisol* (F), *corticosterone* (B) *and aldosterone by the adrenal of the immature pig*

(Figures for aldosterone corrected for losses; all samples of pig I collected from the left adrenal for 30 min., of pig IV from both adrenals after evisceration for 10 min.)

Pig no. sex and race	Body weight (kg.)	Age (weeks)	Blood sample no.	Secretion rates: Cortisol + corticosterone (μg./g. adrenal/hr.)	Cortisol + corticosterone (μg./l adrenal/hr.)	Ratio F/B	Aldosterone (μg./g. adrenal/hr.)	Aldosterone (μg./l adrenal/hr.)	Mean blood pressure (mm. Hg)	Adrenal blood haematocrit (% red cells)	Dextraven infused (ml./kg.)
I, male, Landrace	14	7	S_1	1226	734	2·3	12·9	7·7	115	—	0
			S_2	1271	761	3·0	11·4	6·8	115	—	0
			S_3	1270	758	1·9	14·0	8·4	105	—	3·6
			S_4	1186	710	1·9	12·7	7·6	100	—	1·4
IV, female, Large White	13	9	S_1	1844	1566	1·1	26·1	22·2	50	42	0
			S_2	1108	942	1·1	20·1	17·1	40	44	0
			S_3	945	804	1·4	8·8	7·5	30	35	7·3
			S_4	1115	948	1·1	13·4	11·4	40	20	5·0
			S_5	1496	1272	1·0	20·5	17·4	55	15	4·6
			S_6	1374	1170	1·2	16·6	14·2	65	12	4·6
			S_7	1242	1056	1·5	10·6	9·0	50	10	2·3
			S_8	1058	900	1·4	12·0	10·2	45	8	2·3

androgen could no longer be detected 2 hr. after removal of the pituitary. Large amounts of cholesterol were still present in the adrenal venous and arterial blood 2 hr. after hypophysectomy.

A summary of the quantitative estimations is given in Table 4. In most cases the amounts were too small to measure, as the limit of sensitivity was about 0·2 μg./sample. For purposes of comparison, the Table also includes observations on glucocorticoid secretion rates before and after hypophysectomy.

Table 4. *Adrenal secretion of 11βOH-androstenedione and of pregnenolone, progesterone and 11βOH-progesterone in dogs before and 2 hr. after hypophysectomy in comparison with the secretion of glucocorticoids (no correction for losses)*

					Secretion rates (μg./adrenal/hr.)				
Dog no.	Sex	Body weight (kg.)	State of pituitary	Sample no.	11βOH-Androstenedione*	Pregnenolone*	Progesterone*	11βOH-Progesterone*	Cortisol+ticosteron
1	F‡	11·0	Intact	1	19·2	23·0	8·0	20·4	—
2	M	15·2	Intact	1	6·4	9·4	12·3	27·0	—
3	F	15·0	Intact	1	11·8	26·5	20·0	28·0	1465
4	M‡	11·3	Removed	1	< 0·5	< 0·5	< 0·5	< 0·5	58
5	M‡	10·0	Removed	1	< 0·5	< 0·5	< 0·5	< 0·5	102
6	M‡	10·0	Removed	1	< 0·5	< 0·5	< 0·5	< 0·5	46
7	M	16·5	Intact	1	30·6	44·0	4·0	7·0	924
			Removed	2	1·7	< 0·4	< 0·4	< 0·5	160
8	F	13·8	Intact	1	11·0	8·0	8·6	26·2	952
			Removed	2	< 0·2	< 0·2	< 0·5	< 0·2	30
9	M	18·0	Intact	1	25·0	25·8	21·0	36·0	1428
			One half removed	2	13·0	—	16·0	27·0	782

* Assay by gas–liquid chromatography.
† Assay by reaction with blue tetrazolium.
‡ Bilateral nephrectomy.

In dog no. 9, hypophysectomy was only partial and glucocorticoid secretion was reduced by one half. The secretion rate of the androgen 11βOH-androstenedione fell also by 48%. A somewhat smaller decrease (25%) occurred in the secretion of progesterone and 11βOH-progesterone. Pregnenolone was not measured after hypophysectomy in this dog.

DISCUSSION

In the experiments described, observations were made simultaneously on the adrenal secretion of androgens and of several intermediates in the biosynthesis of corticosteroids. A detailed examination of adrenal blood extracts was carried out by combining older methods of steroid analysis with gas chromatographic techniques. In this way most of the first 26 steroids listed in Table 1 could have been detected in any given sample, had they been present in an unconjugated form and in quantities of more than 0·2 μg. By gas chromatography, substantial amounts of a number of compounds which were retained longer than cholesterol were also detected; reference compounds with similar properties have not yet been tested. It is possible that they include non-steroidal compounds or metabolites of cholesterol or not yet identified. Although most of the known steroids which are less polar than

corticosterone are easily detectable by GLC under the conditions employed, at least one compound failed to give a detector response: a u.v. light-absorbing band was visible in the pregnenolone region of the paper chromatogram; when the eluate of this region was subjected to GLC only one peak corresponding to pregnenolone was obtained.

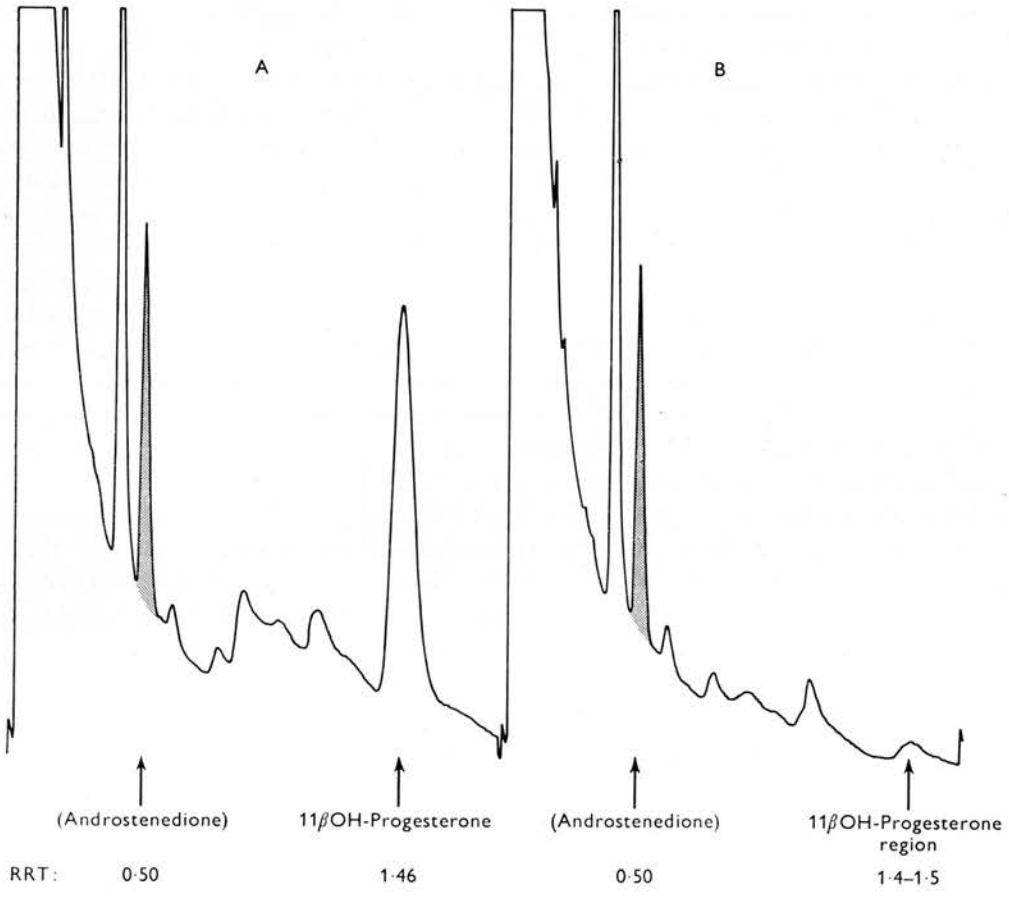

Fig. 4. Gas chromatographic evidence for the effect of hypophysectomy on the secretion of 11βOH-progesterone (dog no. 7). Adrenal venous blood collected for 30 min. periods (A) before and (B) 2 hr. after hypophysectomy. Blood extracts chromatographed for 3 hr. in system B_3. Dried eluates of the 11βOH-progesterone regions dissolved in 30 μl. ethanol containing 3 μg. androstenedione (reference steroid for quantitation, shaded peaks). Gas chromatographic tracings from 1 μl. aliquots (range 1, attenuation 16). Peaks with an RRT (relative to cholestane) of 0·39 = 'paper blanks'; RRT of 0·5 = androstenedione; RRT of 1·45 = 11βOH-progesterone.

A reliable quantitative estimation of steroids by GLC was achieved by the addition of a known amount of a suitable reference steroid and by relating the size of the detector response obtained from the steroid to be assayed to that from the reference steroid. The sensitivity of this method was high (2–10 ng.) when pure steroids were tested. For biological work the limit reached was 200 ng./sample because impurities restricted the size of the aliquot of each extract which could be subjected to GLC. The precision of the method for the estimation of pure steroids dissolved in ethanol was

high. No detailed analysis of errors has yet been made on biological extracts but duplicate assays on some blood extracts indicate that an estimated difference of 30% in the amount of a steroid present in two samples can be regarded as significant. In order to study steroids which are secreted in smaller amounts, the extracts will have to be further purified and modifications of the gas-chromatographic method introduced.

In the adrenal venous blood of stressed pigs and dogs, in addition to glucocorticoids and aldosterone, the following four steroids were identified as regular secretion products: 11βOH-androstenedione, pregnenolone, progesterone and 11βOH-progesterone. The absence ($<$ 200 ng./100 ml.) of these four steroids in arterial blood of the same animals collected at the same time established that they were secreted by the adrenal gland. The 11β-hydroxylated compounds have been found previously in adrenal blood of dogs (Oertel & Eik-Nes, 1962), and 11βOH-androstenedione and progesterone in that of a pig (Balfour, Comline & Short, 1957; Short, 1960).

The only adrenal androgen which was found to be secreted by all the experimental animals was 11βOH-androstenedione. It was secreted at a mean rate of approximately 17 μg./adrenal/hr. in both species; this is about one-fiftieth of the secretion rate of glucocorticoids and approx. twice that of aldosterone under the same conditions (Holzbauer, 1964). The presence of very small amounts of androstenedione, adrenosterone, and 16αOH- and 17αOH-progesterone was sometimes indicated. Owing to technical difficulties no definite information could be obtained about free DHA. 17αOH-Pregnenolone was not studied since it had an R_F value similar to aldosterone, an area of the paper chromatogram not examined by GLC. Carstensen, Oertel & Eik-Nes (1959) have reported that this steroid is secreted in significant amounts by the canine adrenal gland during stimulation with exogenous ACTH (about 10 m-u./kg./min.) but it was not detected under conditions of anaesthetic stress.

The observations on hypophysectomized dogs provided direct evidence for pituitary control of adrenal androgen secretion. The absence of detectable amounts of 11βOH-androstenedione from adrenal venous blood 2 hr. after hypophysectomy indicates a fall by more than 90%; this was coincident with a decrease by more than 90% in glucocorticoid secretion. In dog no. 9, in which only part of the pituitary was removed, the secretion rates of both 11βOH-androstenedione and of the glucocorticoids were reduced by about 50%. These findings suggest a constant secretion ratio of the two types of steroids under different conditions. If this were correct, androgen secretion under resting conditions would be negligible, because the glucocorticoid secretion of the unstressed dog (Nelson, Egdahl & Hume, 1956) is as low as that found 2 hr. after hypophysectomy. This possibility raises the question whether a physiological function can be ascribed to adrenal androgens, or whether the suggestion of Short (1960) that the secretion of sex hormones by a hyperactive adrenal is accidental, has to be accepted. The possibility that the increased output of 11βOH-androstenedione, observed in all stressed animals to be at a fairly similar rate, has a beneficial effect on the organism cannot be ruled out, although there is, as yet, no proof for such a view.

Pregnenolone, progesterone and 11βOH-progesterone, which are key intermediates in adrenal steroid synthesis, were also found to be regularly secreted during periods of increased ACTH release and at rates similar to those of 11βOH-androstenedione.

In the absence of the pituitary gland they could not be detected. The secretion of these precursors under conditions of a highly accelerated steroid biosynthesis may represent an overspill from the cells as the enzyme systems become saturated with substrate.

Corticosteroid secretion in the immature pig was similar to that in the adult dog, cortisol being the major glucocorticoid. Whether the difference in the ratio cortisol : corticosterone between pig II and pig IV (Table 3) was due to the difference in race, sex and/or operative procedures will require further investigation. The rate of secretion of aldosterone was of the same order as in the dog under similar experimental conditions. At the very low blood pressure at which adrenal blood was collected in pig IV a positive correlation existed between steroid secretion rates and arterial blood pressure. It is of interest to note that steroid secretion was hardly affected by the dramatic loss of red cells during the course of this experiment.

Our thanks are due to Professor W. Klyne (M.R.C. Steroid Reference Collection) for samples of authentic steroids and to Benger Laboratories for a gift of Dextraven. We are indebted to Dr M. Vogt, F.R.S., for encouragement and for her interest in this work. We acknowledge the help of Mr R. Hughes during the operative work and the solvent preparation and thank Miss Valerie Wilson for technical assistance.

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EVIDENCE FOR THE PRESENCE OF 16α-HYDROXYPREGN-4-ENE-3,20-DIONE IN ADRENAL VENOUS BLOOD OF YOUNG PIGS

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SUMMARY

1. Evidence was obtained for the presence of 16α-hydroxypregn-4-ene-3,20-dione in each adrenal venous blood sample collected from eight young pigs under conditions of operative stress.

2. The chemical identity of this steroid was tested on μg amounts by paper and gas chromatographic methods before and after the formation of derivatives.

INTRODUCTION

During studies of the adrenal steroids which are secreted by domestic animals, small amounts of a compound with chromatographic properties similar to those of 16α-hydroxypregn-4-ene-3,20-dione (16α-OH-progesterone) were found in the adrenal vein blood from a young pig (Heap, Holzbauer & Newport, 1966).

This compound has now been detected in fifteen adrenal blood samples collected from another eight pigs and further evidence for its identity with 16α-OH-progesterone has been obtained.

METHODS

Female Landrace or Large White pigs, 8–12 weeks old, were premedicated with Sernylan (Parke, Davis and Co., 1-(phenyl-cyclohexyl) piperidine HCl, 1 mg/kg body weight, subcutaneous) and when sedated they were anaesthetized with halothane followed by chloralose (Merck, 40 mg/kg body weight, 0·7 % solution in 0·9 % NaCl, I.V.). An abdominal mid line incision was made and the viscera and both kidneys were removed. The region of the inferior vena cava in the proximity of the adrenal glands was freed from surrounding tissue. Venous tributaries not coming from the adrenals were tied. The abdominal aorta and cava were ligated below the renal vessels and a cannula was inserted into the cava above this ligature. The vena cava was occluded at its entry into the liver and the venous blood from both adrenals was collected in a cooled glass cylinder. Collection periods were either 5 or 10 min. Both adrenals were removed from the animal immediately after the collection of the blood.

The blood samples and the adrenals were extracted and the extracts purified as described previously (Holzbauer, 1957, 1964). All samples were further purified and analysed by paper and gas-liquid chromatography. The paper used was Whatman no. 50. It was first treated with 2N-NaOH followed by distilled water (Sharman, 1963) and then washed with the mixture used to elute the chromatograms (ethyl acetate:methanol, 2:1) and the mobile phase of Bush's system B_3 (Bush, 1952). This purification procedure greatly reduced non-specific peaks ('paper blanks') which otherwise occurred on the gas chromatographic record when eluates from papers were applied. Furthermore, steroid spots on chromatograms developed on NaOH-pretreated Whatman no. 50 paper showed much less tailing than on untreated Whatman no. 2 paper.

Gas-liquid chromatography was carried out on a Model 402 F & M gas chromatograph using a 4 ft. (122 cm) 3·8 % SE-30 column at 230° C, a flame ionization detector and argon as carrier gas. The retention times of the steroids were expressed in relation to that of cholestane which averaged 20 min, and they are referred to as relative retention times. The dried eluates from paper chromatograms were dissolved in 40 μl. of ethanol and volumes of 1–2 μl. applied to the gas-liquid chromatograph. All solvents used were specially purified (Heap *et al.* 1966).

RESULTS

The purified blood extracts were first chromatographed for 4 hr in the E_2B system of Eberlein & Bongiovanni (1955) to separate off the major glucocorticoids. In this system, 16α-OH-progesterone has an R_F value of 0·85. The eluates of these regions were rechromatographed for 3 hr in the B_3 system to separate 16α-OH-progesterone from other C_{21} and C_{19} steroid.

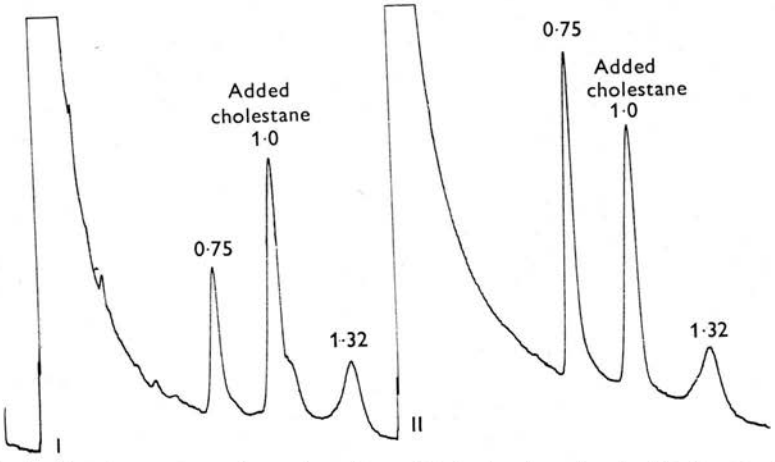

Fig. 1. Gas chromatograph tracings. I: purified pig adrenal vein blood extract, rechromatographed in B_3 (24 hr). U.V. absorbing spot with R_F value equal to that of authentic 16α-OH-progesterone was eluted, dried, dissolved in 40 μl. ethanol, 1 μl. applied to the gas-liquid chromatograph (range 1, attenuation 8). (Shoulder on cholestane peak due to 'paper blank', relative retention time 1·04.) II: authentic 16α-OH-progesterone. The figures above each peak give the retention times relative to that of cholestane.

Provisional evidence was obtained by gas-liquid chromatography for the presence of 16α-OH-progesterone in each individual sample.

For further identification the eluates of eight blood samples were combined and then divided into three equal parts.

One-third was rechromatographed in the B_3 system for 24 hr to separate 16α-OH-progesterone from 17α,21-dihydroxypregn-4-ene-3,20-dione (compound *S*). Two U.V. absorbing spots were visible with R_F values corresponding to those of authentic 16α-OH-progesterone and compound *S*. The 16α-OH-progesterone region was eluted.

When authentic 16α-OH-progesterone was applied to the gas chromatograph two peaks were consistently observed with relative retention times of 0·75 and 1·32 (Fig. 1, II). The first peak was always larger than the second one. The peak size ratio varied which makes it unlikely that one of the peaks is due to an impurity. It is more likely that the steroid is split

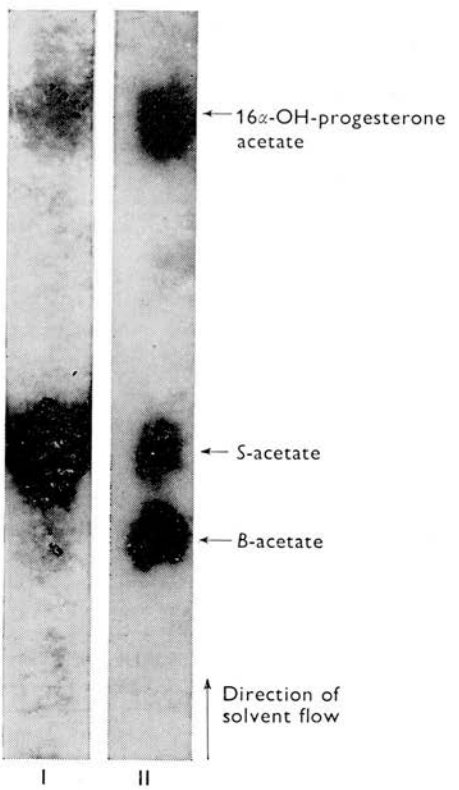

Fig. 2. Contact photograph of U.V. absorbing spots on a paper chromatogram developed for 3½ hr in B_3. I: partially purified 16α-OH-progesterone fraction from pig adrenal vein blood, acetylated. II: authentic steroid acetates.

into these two compounds on the column of the gas chromatograph. The eluate of the 16α-OH-progesterone region of the paper chromatogram produced on the gas-chromatographic records two peaks with the same relative retention times as the authentic steroid (Fig. 1, I). Cholestane was injected simultaneously to enable precise assessment of the relative retention times.

The second part of the combined sample was evaporated to dryness, acetylated (Holzbauer & Vogt, 1961) and chromatographed in the B_2 system for 3½ hr. Authentic 16α-OH-progesterone (20 μg) and compound *S* (20 μg) were treated in the same way. The lane to which the acetylated blood extract was applied showed two U.V. absorbing spots in positions corresponding to those of compound *S* acetate and 16α-OH-progesterone acetate on the control lane (Fig. 2). Both the authentic 16α-OH-progesterone acetate and the corresponding region of the chromatogram of the blood extract were eluted and subjected to gas-liquid chromatography. Each eluate produced two peaks with relative retention times of 0·72 and 1·75 and a small peak at 1·04 corresponding to the 'paper blank' (Fig. 3). Like the free steroid, the acetate produced two peaks on the gas chromatograph tracing. In this case the compound which was retained longer was the

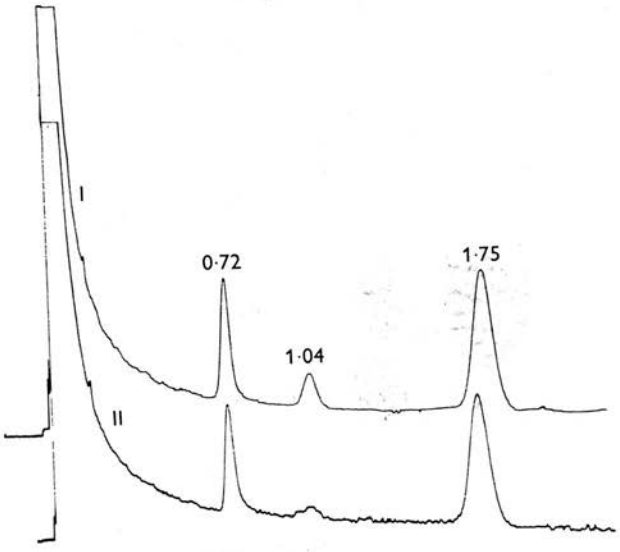

Fig. 3. Gas chromatograph tracings. I: eluate of U.V. absorbing spot with R_F equal to that of 16α-OH-progesterone acetate from lane I, Fig. 2. II: eluate of authentic 16α-OH-progesterone acetate, lane II, Fig. 2. Relative retention time 1·04: 'paper blank'. Both eluates dried, dissolved in 40 μl. ethanol, 1 μl. portions applied to the gas-liquid chromatograph (range 1, attenuation 8). The figures above each peak give the retention times relative to that of cholestane.

dominant one, but again the peak size ratio varied from run to run, whereas the relative retention times remained precisely reproduceable. The first peak was not due to 16α-OH-progesterone which had escaped acetylation as this would not have occurred in the same region of the paper chromatogram as the acetate. It is possible, however, that the breakdown product formed on the column in the gas chromatograph with a relative retention time of 0·72 was similar to that compound which produced a peak with a relative retention time of 0·75 when the free steroid was applied.

Further evidence for the identity of the substance in question with 16α-OH-progesterone was obtained by its conversion into 16α,20β-dihydroxypregn-4-en-3one diacetate. For this purpose the last third of the combined sample was incubated with the enzyme 20β-hydroxysteroid dehydrogenase (Boehringer, Mannheim) under conditions in which it reduces a 20-keto group to a 20β-hydroxyl group (Henning & Zander, 1962; Heap, 1964). Authentic 16α-OH-progesterone (20 μg) was treated in the same way. Subsequently the purified extracts of the incubation media were acetylated and chromatographed for 6 hr in the E_1 system of

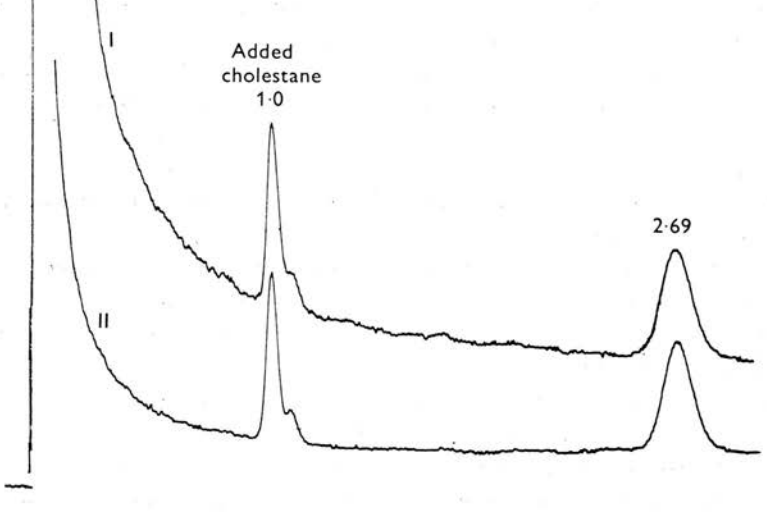

Fig. 4. Gas chromatograph tracings. I: partially purified 16α-OH-progesterone fraction from pig adrenal vein blood after incubation with 20β-hydroxysteroid dehydrogenase, acetylation and chromatography in E_1, 6 hr. Eluate of U.V. absorbing spot with R_F equal to that of authentic 16α,20β-dihydroxypregn-4-en-3one diacetate. II: authentic 16α-OH-progesterone after incubation with 20β-hydroxysteroid dehydrogenase, acetylation and chromatography in E_1, 6 hr. (Shoulder on cholestane peak due to 'paper blank'.) Both eluates dried, dissolved in 40 μl. ethanol, 1 μl. portions applied to the gas-liquid chromatograph (range 1, attenutation 8). The figures above each peak give the retention times relative to that of cholestane.

Eberlein & Bongiovanni (1955). Both the incubated authentic steroid and the blood extract showed a U.V. absorbing spot at R_F 0·5. These regions were eluted and the eluates subjected to gas-liquid chromatography (Fig. 4). Each eluate produced only one peak with a relative retention time of 2·69 in addition to the small 'paper blank' peak (relative retention time 1·04).

The combined extracts of eight pig adrenals were tested in an equal fashion for the presence of 16α-OH-progesterone and the results were the same as those obtained with the blood extracts.

DISCUSSION

The results of the present experiments are evidence for the presence of 16α-OH-progesterone in adrenal venous blood which was collected from 8 to 12 week old pigs under conditions of operative stress. It is unlikely that this steroid could have arisen from any source other than the adrenal cortex since no venous blood from organs which are capable of producing C_{21} steroids could return to the circulation. Extracts from the adrenal glands of the same animals also contained 16α-OH-progesterone.

The amounts available were not sufficient for identification by infra-red spectroscopy or by crystallization and estimation of the melting point. However, formation of derivatives and their analysis by gas-liquid chromatography after repeated paper chromatography in different solvent systems leaves little doubt of the chemical identity of the compound with 16α-OH-progesterone. Its rate of secretion was of the order of 10–50 μg/g adrenal/hr and the amount extracted from 10 g adrenal tissue was about 20 μg. Accurate quantitative estimation of 16α-OH-progesterone by gas-liquid chromatography, complicated by its splitting into two compounds, might be achieved by forming the 20β-hydroxy derivative, which, in its free form, has a relative retention time of 1·51 and appears to be stable on gas-liquid chromatography.

Wettstein, Neher & Urech (1959) isolated a small amount of 16α-OH-progesterone from pig adrenals. The ability of the human adrenal gland to effect 16α-hydroxylation of progesterone *in vivo* was demonstrated by Bird Wiqvist, Diczfalusy & Solomon (1966) who infused [^{14}C]progesterone into intact human foetuses and found 16α-OH-progesterone in the blood of the umbilical artery. This steroid was no longer formed when the foetuses were adrenalectomized (Wilson, Bird, Wiqvist, Solomon & Diczfalusy, 1966).

On the other hand, no 16α-OH-progesterone was found by us (to be published) in the adrenal venous blood collected under similar conditions from three 10-week-old puppies of both sexes; neither was it detected in the adrenal tissue of these animals.

The biological significance of the secretion of 16α-OH-progesterone by the adrenal of young pigs under stress conditions remains at present obscure.

Our thanks are due to Professor W. Klyne (M.R.C. Steroid Reference Collection) for a sample of crystalline 16α-OH-progesterone, to Dr R. B. Heap for his help with the enzyme incubation and to Mr R. Hughes for his technical assistance.

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Secretion of Pregn-4-ene-3,20-dione (Progesterone) *in vivo* by the Adrenal Gland of the Rat

Feder, Resko and Goy[1] suggested recently that the adrenal gland of the rat secretes progesterone. They observed that the arterial blood of female rats still contained significant amounts of progesterone 25 days after bilateral ovariectomy; 8 h after additional adrenalectomy, however, the steroid was no longer detectable. Because the rat is often used for experiments in reproductive physiology, it is important to assess the contribution of the adrenal gland to the total available progesterone in the female rat. We have therefore measured the secretion of progesterone from the adrenal gland by estimating the amount of the steroid secreted into the adrenal venous blood. Experimental conditions were maintained comparable with those used recently for measuring the secretion of progesterone by the ovary of the rat[2–4].

Virgin female Wistar rats, 6 weeks (110–130 g) or 9 weeks (170–230 g) old, were used. Venous blood was collected from the left adrenal gland under pentobarbitone sodium anaesthesia (50 mg/kg) for 30 min, using the method described by Vogt[5]. Results are included from rats with adrenal glands which had been demedullated for other reasons 19–44 days before the adrenal blood was collected. Blood pressure was recorded from a carotid artery and supported by infusing either rat blood or a 0·9 per cent solution of sodium chloride. Before adrenal vein cannulation the arterial pressure ranged from 113 to 165 mm Hg; when blood was being collected it was lower (65–100 mm Hg). There was no significant difference between rats with intact or demedullated adrenal glands. In 30 min 2–3 ml. of adrenal blood was collected. To correct for losses during the chemical procedures 4-^{14}C-progesterone (0·4 mμCi) was added to each blood sample. The whole blood was extracted with a mixture of ethylacetate and ether (2 : 1, v/v), purified and applied to 2 cm wide strips of Whatman No. 2 chromatography paper. A descending chromatogram was developed for 40 h at 27° C in the toluene-propylene glycol (TPG) system of Zaffaroni and Burton[6]. The overflow which contained the progesterone was rechromatographed on sodium hydroxide-washed Whatman No. 50 paper in the E_1-system of Eberlein and Bongiovanni[7]. Progesterone was identified and estimated by gas–liquid chromatography in eluates from the E_1-chromatogram as described before[8]. Corticosterone was estimated according to its reaction

Table 1. SECRETION OF PREGN-4-ENE-3,20-DIONE (PROGESTERONE) BY THE ADRENAL GLAND OF THE RAT

Rat No.	Left adr. gland (mg)	Steroid secretion rates: Progesterone (μg/left adr./h/ 100 g b.wt.)	Steroid secretion rates: Progesterone (μg/h/ 100 mg adr.)	Steroid secretion rates: Corticosterone (μg/h/ (100 mg adr.)	Phase of oestrous cycle
Adrenal glands intact					
C1	25·4	0·28	2·13	144	—
C2	25·2	0·17	1·11	117	—
C3	26·0	0·26	1·69	86	Pro-oestrus
C4	35·8	0·61	3·24	196	Oestrus
C6	31·3	0·16	1·02	76	Oestrus
C7	15·9	0·23	1·89	61	Pro-oestrus
C8	21·7	0·43	2·49	115	Oestrus
	Mean ± *S.E.*:	0·31 ± 0·06	1·94 ± 0·29	114 ± 17	
Adrenal glands demedullated					
D1	13·4	0·07	1·00	65	—
D2	25·4	0·72	4·88	293	—
D3	25·6	0·16	1·25	173	Oestrus
D4	34·2*	0·28	1·64	174	Met-oestrus
D5	25·6	0·44	3·59	149	Met-oestrus
D6	30·0	0·31	2·33	191	Vagina artretic
D7	6·8	0·12	2·06	44	—
D8	10·4	0·16	1·92	204	Met-oestrus
	Mean ± *S.E.*:	0·28 ± 0·08	2·33 ± 0·46	162 ± 28	

	Left ovary mean: (mg)	Ovarian progesterone secretion rates† Progesterone secretion rates (μg/left ovary/h/ 100 g b.wt.) (mean ± *S.E.*)	(μg/h/ 100 mg ovary) (mean ± *S.E.*)	(Range)	
n = 8	38·8	1·81 ± 0·59	9·35 ± 3·9	2·16 – 33·30	Oestrus, met-oestrus
n = 4	36·8	< 0·11	< 0·52		Early pro-oestrus

Virgin female Wistar rats, 6 weeks (C1–C6, D1–D6, 110–130 g b.wt.) or 9 weeks (C7, C8, D7, D8, 170–230 g b.wt.); venous blood was from the left adrena gland collected under pentobarbitone sodium anaesthesia between 1000 and 1500 for periods of 30 min; body temperature was 37–37·8° C; C; control rats; D: rats with demedullated adrenal glands. Days after demedullation: D1, D2: 37; D3, D4: 43; D5, D6: 44; D7, D8: 19; —: not recorded; adr.: adrenal gland; b.wt.: body weight.

* Right adrenal removed.

† Results obtained in previous experiments on rats of the same strain (mean b.wt. 180 g) in similar experimental conditions[3].

with tetrazolium blue on eluates of the corresponding regions of the TPG chromatogram.

The results are given in Table 1. Progesterone was detected in the adrenal venous blood of all rats studied, except rat D1. The concentrations of progesterone in the adrenal venous blood varied between 0·04 and 0·15 μg/ml. Progesterone could not be detected in 5 ml. of arterial blood (< 0·03 μg/ml.) by the method used. The rates of secretion of corticosterone were of the same order of magnitude as those found earlier[9] and about fifty times greater than the secretion of progesterone.

Mean values of rates of ovarian progesterone secretion, obtained by Fajer and Holzbauer[3], who collected ovarian venous blood from rats of the same strain in similar experimental conditions, are included in Table 1. Comparison of these ovarian secretion rates of progesterone with adrenal progesterone secretion shows that, in conditions of surgical stress, the adrenal gland contributes a large proportion of the total progesterone available to the female rat. These results agree with the observations and conclusions of Feder *et al*[1].

The pig is another animal in which the adrenal gland can contribute a considerable proportion of the progesterone available to the body[10]. The adrenal secretion of progesterone has been found to vary between 0·2 and 3 μg/min. In similar conditions of stress, the secretion of progesterone from the ovaries during the oestrous cycle was reported to vary from 0·6 to 30 μg/min[11].

Further experiments were carried out with rats subjected to adrenal demedullation, for the pattern of steroid secretion might be modified by this operation. The results show that the adrenal secretion of corticosterone and progesterone was of the same magnitude in the absence of the adrenal medulla.

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QUANTITATIVE ESTIMATION OF 17α-HYDROXYPREGN-4-ENE-3,20-DIONE (17αOH-PROGESTERONE) IN ADRENAL VENOUS BLOOD AND ADRENAL GLANDS

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SUMMARY

1. A method is described for the accurate estimation of small quantities of 17α-hydroxypregn-4-ene-3,20-dione (17αOH-progesterone) in blood and adrenal glands.

2. The dog adrenal was found to secrete 17αOH-progesterone under conditions of operative stress at a rate of 5–10 μg/g tissue/hr. This is similar to the secretion rate of aldosterone.

3. The secretion of 17αOH-progesterone was decreased after hypophysectomy and increased in response to adrenocorticotrophic hormone (ACTH).

4. The adrenal gland of the dog, the monkey and the guinea-pig was found to contain between 0·6 and 6 μg of 17αOH-progesterone/g tissue. The concentration was usually somewhat smaller than that of pregnenolone or progesterone and between 1 and 20 % of the concentrations of the glucocorticoids.

5. A comparison of adrenal steroid contents and secretion rates under conditions of stress indicates a rate of synthesis in the order of 30–40 n-moles of 17αOH-progesterone/g adrenal tissue/min.

INTRODUCTION

17αOH-Progesterone occupies a key position in the synthetic pathway which leads to the formation of cortisol and cortisone in the adrenal gland. In addition, it is an intermediate in the biosynthesis of androstenedione in the adrenal gland (Cohn, 1965) and in the ovary (Aakvaag & Eik-Nes, 1965). Biological activity of this steroid has been described by Jacobs, van der Poll, Gabrilove & Soffer (1961), who observed sodium loss in normal human subjects after its administration.

The presence of 17αOH-progesterone in adrenal venous blood and

adrenal tissue has been demonstrated in several species (for references see Dorfman & Ungar, 1965). However, quantitative studies have so far not been carried out because no accurate sensitive method was available for its estimation. In the present paper a method is described which allows the estimation of small quantities of this steroid in biological material. Some observations on the adrenal synthesis and secretion of 17αOH-progesterone are reported.

METHODS

Operative procedures

Adult dogs or puppies (10 weeks old) were anaesthetized either with chloralose (70 mg/kg body wt.) or pentobarbitone sodium (5 % solution, 8 ml./kg body wt.). Adrenal venous blood was collected for periods of 30 min as described previously (Holzbauer, 1964). Hypophysectomy was carried out through the roof of the mouth. The adrenal glands were removed immediately after death.

Chemical procedures

Extraction and purification. Whole blood was extracted immediately after each collection period and the extracts purified as described by Holzbauer (1964). The adrenal glands were extracted immediately after removal from the body or kept at $-17°$ C. They were homogenized in a glass homogenizer, using a mixture of ethyl acetate and water 2:1, the homogenate was centrifuged and the bottom aqueous layer was re-extracted with an equal volume of ethyl acetate. The addition of water facilitated the breaking up of the tissue. The combined ethyl acetate extracts were purified by the same procedure as the blood extracts. [4-^{14}C]-17αOH-progesterone (5 nc) was added to the whole blood before extraction and to the homogenates of the gland tissue before centrifugation. Descending paper chromatograms were developed in the E_2B and the E_1 systems (Eberlein & Bongiovanni, 1955) and the B_3 system (Bush, 1952) at 28° C. For the E_2B system Whatman No. 2 paper was used after it had been washed with the mixture used for eluting steroids from the paper (ethyl acetate : methanol = 2:1 v/v). For chromatography in the E_1 and B_3 systems Whatman No. 50 paper was used, pretreated with NaOH as described by Sharman (1963). Eluates of paper thus pretreated usually produce only one small peak on gas chromatography.

The conditions for gas liquid chromatography were as follows: Model 402 F & M gas chromatograph, 4 ft (122 cm) 3·8 % SE-30 column at 230° C, flame ionization detection (260° C) and argon as carrier gas. The retention times of the steroids on the column were expressed in relation to that of cholestane (which averaged 20 min) and they will be referred to as relative retention times (RRT). The dried eluates from the paper chromatograms were dissolved in 10–50 μl. of ethanol and quantities of 1–3 μl. applied to the column.

Oxidation. The reagent used was a 0·15 % solution of chromium trioxide (CrO_3) in redistilled glacial acetic acid. Because the CrO_2 dissolves very slowly, the solution was prepared at least 1, but not more than 8, days before use from CrO_3 which had been kept in a desiccator over anhydrous $CaCl_2$. A solution of an authentic steroid, containing 0·1–50 μg steroid, or a paper eluate, was brought to absolute dryness under a stream of nitrogen at 50° C. The residue was dissolved in 0·1 ml. of the CrO_3 reagent and shaken for 20 min at room temperature; 1 ml. of 20 % ethanol was added and the oxidized steroid extracted with 5 ml. dichloromethane.

Reduction. Keto groups in position C-17 were reduced with sodium borohydride ($NaBH_4$) according to Bardin & Peterson (1967). The dried samples were dissolved in 0·5 ml. ethanol, 0·02 ml. of a freshly prepared 2 % solution of $NaBH_4$ in water was added and 20 sec later 3 drops of glacial acetic acid and 2 ml. of water were added. The steroids were then extracted with dichloromethane.

Acetylation. The reaction used was the same as described by Holzbauer & Vogt (1961). Under these conditions aldosterone is transformed into its diacetate.

Chromatogram markers (control spots). Steroids containing a Δ^4-3-keto grouping were located under u.v. light. Some steroids were visualized by their colour reaction with antimony trichloride ($SbCl_3$), the control lanes being dipped into a saturated solution of $SbCl_3$ in chloroform and dried at 80° C for 20 sec.

Estimation. The glucocorticoids and 21-hydroxypregn-4-ene-3,20-dione (DOC) were estimated by their reaction with blue tetrazolium (Vogt, 1955). The remaining steroids were assayed by gas chromatography by the procedure of Heap, Holzbauer & Newport (1966).

Procedure for the estimation of 17αOH-progesterone

On gas chromatography 17αOH-progesterone produced a peak at RRT 1·12 having a shoulder at RRT 1·42 (Fig. 1*a*). The size of this peak was not reproducible because the steroid was partly destroyed under the conditions used. 17αOH-progesterone was therefore

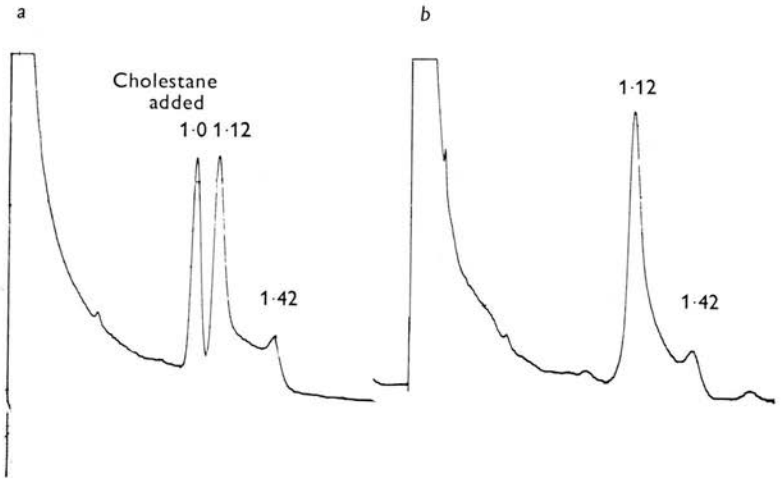

Fig. 1. Gas chromatograph tracings. (*a*) Authentic 17αOH-progesterone (~ 200 ng). (*b*) Extract of 1000 ml. of dog adrenal vein blood. Eluate of 17αOH-progesterone region of B_3 chromatogram evaporated to dryness, dissolved in 40 μl. ethanol, 1 μl. applied to column. The numbers are retention times relative to that of cholestane. Conditions: 3·8 % SE-30 column (122 cm) 230° C; flame ionization detector (260° C); range 1, attenuation 8.

converted to androst-4-ene-3,17-dione (androstenedione) by treating it with CrO_3. Androstenedione gives on gas chromatography a single peak at RRT 0·48 and can be quantitatively estimated by the method described by Heap *et al.* (1966) using androst-4-ene-3,11,17-trione (adrenosterone, RRT 0·58) as reference standard. The principle of this method is based on the straight line relationship between the ratio of the heights of the peaks produced by androstenedione and adrenosterone and the ratio of the concentrations in which the two steroids are present in a solution (Fig. 2, filled circles). When mixtures of oxidized 17αOH-progesterone and adrenosterone were applied to the gas chromatograph the results represented by the open circles in Fig. 2 were obtained. The two calibration lines had the same slope.

The method used for the estimation of 17αOH-progesterone in biological material was as follows. The purified blood or gland extracts were first chromatographed for 4 hr in the E_2B

system. In this system the major glucocorticoids were separated from 17α-OH-progesterone, which travelled near the solvent front together with DOC, androstane derivatives and pregnane derivatives which are not hydroxylated in position C-21. For further isolation of 17α-OH-progesterone the eluate of the front region was chromatographed for 8 hr in the B_3 system. 17α-OH-Progesterone travelled about 27 cm. It was found between DOC and adrenosterone, and was well separated from both of them but it had the same R_F value as testosterone. During the 8 hr androstenedione ran off the paper. The 17α-OH-progesterone region was eluted, the eluate dried, taken up in ethanol and 5–10 % of the solution applied to the gas

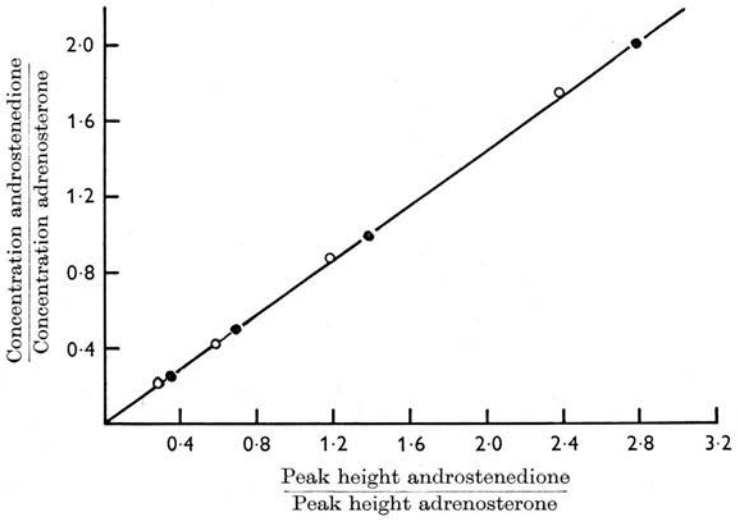

Fig. 2. Calibration line for the gas chromatographic estimation of androstenedione using adrenosterone as reference standard. ●: Mixture of androstenedione and adrenosterone (b = 0·71). ○: Mixture of oxidized 17α-OH-progesterone and adrenosterone (b = 0·73).

chromatograph in order to establish the presence of a peak at RRT 1·12 and the absence of 17β-OH-androst-4-en-3-one (testosterone, RRT 0·53). The rest of the solution was then evaporated to dryness and oxidized with CrO_3. As the conversion of 17α-OH-progesterone to androstenedione is not always complete, paper chromatography in a third system was required, in order to separate the non-oxidized from the oxidized [^{14}C]-labelled steroid. This was accomplished in a 7 hr run in the E_1 system. 17α-OH-progesterone had an R_F value of 0·17 and was well separated from androstenedione (R_F 0·7). The eluate of the androstenedione region was then transferred to 500 μl. pear-shaped glass-stoppered microtubes. The solution was evaporated to dryness, taken up in an appropriate volume (10–100 μl.) of a solution of adrenosterone (100 μg/ml.) and 1–2 μl. portions injected into the gas chromatograph, alternating with standard mixtures of authentic androstenedione and adrenosterone. A portion of the same solution was used for counting the remaining radioactivity in a Tri-Carb liquid scintillation spectrometer. The amount of 17α-OH-progesterone in the original sample was estimated from the amount of androstenedione found in the oxidized sample. The value was corrected for losses as assessed by the isotope counting. The method allows the estimation of as little as 50 ng (= 0·15 n-moles) of 17α-OH-progesterone contained in a sample.

The [4-^{14}C]-17α-OH-progesterone, purchased from the Radiochemical Centre, Amersham,

behaved like a single compound in several paper chromatographic systems. When it was diluted with the authentic compound and oxidized, the specific activity of the purified oxidized product was 20 % lower than that of the original mixture. This different behaviour on oxidation of the labelled and non-labelled steroid was established in a number of experiments and it had to be assumed that only 80 % of the labelled steroid reacted in the same way as authentic crystalline 17αOH-progesterone. The reasons for this discrepancy are under investigation.

Other steroids contained in adrenal blood and tissue extracts which are also oxidized to androstenedione and would interfere with this assay are cortisol, cortisone, 17α, 21-dihydroxypregn-4-ene-3,20-dione (compound S), DOC and testosterone. Cortisol and cortisone were separated off in the E_2B system and compound S in the B_3 system (R_F 0·1). Testosterone, of which less than 2 ng can be detected, has not been found to be present on examination of more than fifty extracts of adrenal blood or glands. The amount of DOC present in dog adrenal blood can be 10 times larger than that of 17αOH-progesterone. Because of the proximity of the two steroids on the first B_3 chromatogram the degree to which DOC could possibly interfere with the estimation of 17αOH-progesterone was tested. In an experiment, in which 8·9 μg of androstenedione was formed from 10 μg 17αOH-progesterone (100 % conversion) only 0·7 μg of androstenedione was formed from 10 μg DOC. Thus, any over-estimation of 17αOH-progesterone due to tailing of the DOC spot would be very small.

Drugs

ACTH. The adrenocorticotrophic hormone (ACTH) used was corticotrophin B.P. (Organon Laboratories Ltd.) for intravenous use, of porcine origin.

RESULTS

One litre of adrenal venous blood of a dog was extracted, purified and 17αOH-progesterone isolated by paper chromatography in the E_2B and B_3 systems. The eluate of the 17αOH-progesterone region was dried and the residue dissolved in 40 μl. ethanol. The picture obtained when 1 μl. of this solution was injected into the gas chromatograph (Fig. 1*b*) was similar to that given by authentic 17αOH-progesterone (Fig. 1*a*). After oxidation of the eluate with CrO_3, the gas chromatogram in Fig. 3 was obtained. The only peak detectable was now a peak at RRT 0·48, the RRT of androstenedione. The identity of this oxidation product with androstenedione was further established by R_F values on paper chromatograms and by the formation of derivatives. In the E_1 system (7 hr) a U.V. absorbing spot was visible with the same R_F value (0·7) as authentic androstenedione. The eluate of this region gave a peak with an RRT of 0·48 on the gas chromatograph. When this eluate was reduced with $NaBH_4$ a compound was formed giving a peak with an RRT of 0·53, the same as that of testosterone, the reduction product which is obtained from authentic androstenedione treated in a similar way. The reduced compound was rechromatographed in the B_3 system (4 hr). It travelled to the same region as testosterone, well separated from androstenedione and also from 17αOH-androst-4-en-3-one (epitestosterone). On acetylation a peak with an RRT of 0·73 was obtained on the gas chromatograph. This is the same as that of testosterone acetate.

When rechromatographed in the E_1 system this acetylated compound travelled to the same position as authentic testosterone acetate ($R_F = 0{\cdot}9$).

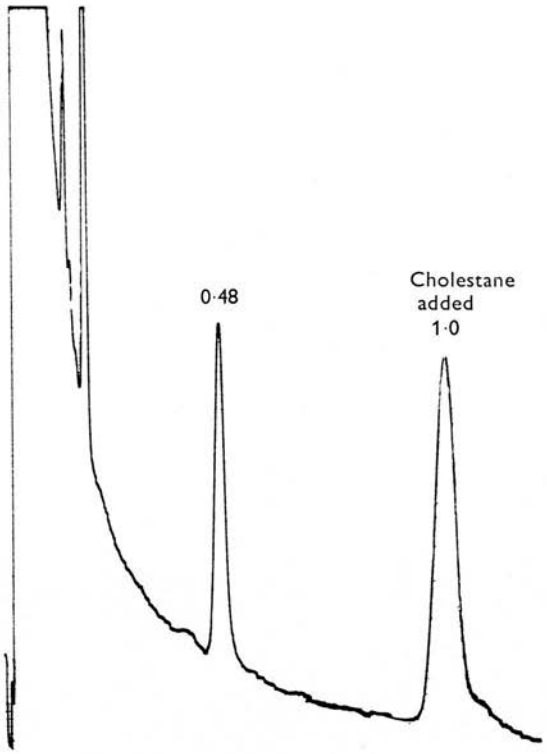

Fig. 3. Gas chromatograph tracing. Extract of dog adrenal vein blood. Eluate of 17αOH-progesterone region of B_s chromatogram (see Fig. 1*b*) oxidized, oxidation products extracted, extract evaporated to dryness, dissolved in 100 μl. ethanol, 1 μl. applied to column. The numbers are retention times relative to that of cholestane (range 1, attenuation 8).

Figure 4 shows results obtained with an extract of a pair of adrenal glands from a monkey. The eluate of the front region of the E_2B chromatogram was rechromatographed in the B_3 system for 8 hr. In the region corresponding to the R_F value of authentic 17αOH-progesterone a U.V. absorbing spot was visible (Fig. 4*a*). This region and the two adjacent regions were eluted and a portion corresponding to 1/20th of each eluate was injected into the gas chromatograph together with 100 ng of cholestane. The tracings obtained are shown in Fig. 4*b*. Only the eluate of the 17αOH-progesterone region (II) showed a peak with an RRT of 1·12. No peaks were visible with an RRT of 0·48 (androstenedione) or 0·53 (testosterone). All three eluates were subsequently oxidized. The gas chromatograph tracings obtained with 1/20th of the oxidation products are shown in

Fig. 4*c*. In the eluate of the 17α*OH*-progesterone region (II) the peak with an RRT of 1·12 has now disappeared and a large peak with an RRT of 0·48 (similar to that of androstenedione) has taken its place. In eluate III there was a very small peak with an RRT of 0·48. The discrepancy in the sizes of the steroid peaks before and after oxidation is explained by the instability of 17α*OH*-progesterone under the conditions used for gas chromatography.

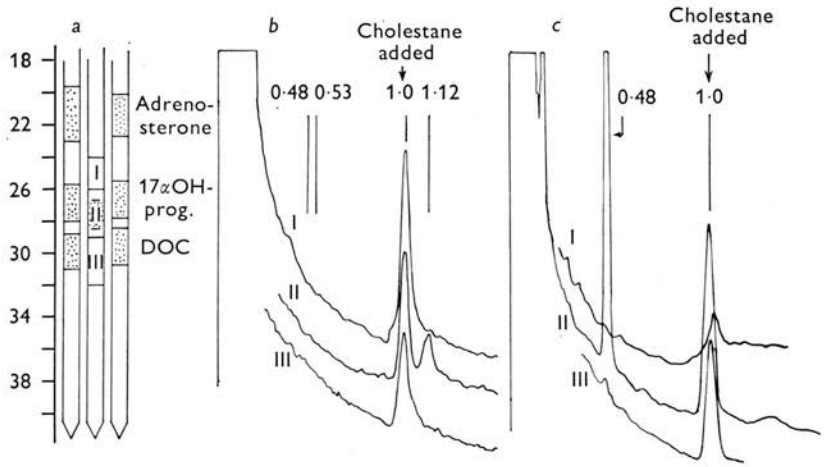

Fig. 4. Analysis of monkey adrenal extract. (*a*) Paper chromatogram developed in the B_3 system for 8 hr. Scale = cm from origin. All lanes 1 cm wide. Outside lanes: control spots. Middle lane: purified extract of 2 monkey adrenals (650 mg). Stippled areas were absorbing U.V. light. (*b*) Gas chromatograph tracings of eluates of paper regions I, II and III, superimposed. All eluates evaporated to dryness, dissolved in 10 μl. ethanol, 1 μl. applied to column (range 1, attenuation 8). (*c*) Gas chromatograph tracings of the oxidation products: the remaining ethanol was evaporated, the residues treated with chromium trioxide, the oxidation products extracted, the extracts dried, dissolved in 10 μl. of ethanol and applied to the column (range 1, attenuation 8). The numbers are retention times relative to that of cholestane. II: 17α*OH*-progesterone region. I and III: regions adjacent to 17α*OH*-progesterone region.

The results of quantitative analyses of 17α*OH*-progesterone in samples of adrenal venous blood which were collected from a dog before and after hypophysectomy and during an infusion of ACTH are listed in Table 1. ACTH was infused at a rate which had previously been found to restore glucocorticoid secretion in the acutely hypophysectomized dog to pre-hypophysectomy rates (Holzbauer, 1964). For purposes of comparison the secretion rates of progesterone and its other two metabolites, 11β*OH*-progesterone and DOC are included, as well as those of the major glucocorticoids. The secretion rate of 17α*OH*-progesterone before hypophysectomy (0·12 μg/g adrenal/min) was similar to that of aldosterone under

TABLE 1. Secretion of 17α-OH-pregn-4-ene-3,20-dione (17α-OH-progesterone), pregn-4-ene-3,20-dione (progesterone), 11β-OH-pregn-4-ene-3,20-dione (11β-OH-progesterone) and 21-OH-pregn-4-ene-3,20-dione (DOC) and the major glucocorticoids by the left adrenal of an adult dog (♀, 20 kg) under chloralose anaesthesia. Adrenal venous blood collected for periods of 30 min from the cannulated left adreno-lumbar vein. The blood withdrawn was replaced by donor blood

Experimental conditions	Blood collected in 30 min (ml.)	Adrenal secretion rates (nanomoles/g adrenal/min)				Compounds†		Adrenal blood concentration (n-moles/100 ml.)
		17α-OH-progesterone (mol. wt. = 330·5)	Progesterone (mol. wt. = 314·5)	11β-OH-progesterone (mol. wt. = 330·5)	DOC (mol. wt. = 330·5)	*F*+*E*+*S*	*B*+*A*	17α-OH-progesterone
Pituitary intact; 30 min after cannulation of adrenal vein	130	0·36	0·90	3·64	2·10	43·2	18·1	6·45*
1 hr after hypophysectomy	120	0·05	0·13	0·26	0·71	11·9	5·8	0·94
During infusion of ACTH (0·3 m-u./min/kg)	103	0·25	0·66	3·53	1·61	40·4	19·3	5·60
After ACTH infusion (2½ hr after hypophysectomy)	107	0·05	0·16	0·33	1·10	10·1	6·3	1·03

* Concentration of 17α-OH-progesterone in blood from femoral artery collected simultaneously: 0·75 n-moles/100 ml.

† Compounds

S: 17,21-dihydroxypregn-4-ene-3,20-dione (mol. wt. = 346·5) *B*: 11,21-dihydroxypregn-4-ene-3,20-dione (mol. wt. = 346·5)

F: 11,17,21-trihydroxypregn-4-ene-3,20-dione (mol. wt. = 362·5) *A*: 21-hydroxypregn-4-ene-3,11,20-trione (mol. wt. = 344·4)

E: 17,21-dihydroxypregn-4-ene-3,11,20-trione (mol. wt. = 360·4)

comparable conditions (Holzbauer, 1964). 17αOH-progesterone was secreted at a much slower rate than the other two progesterone metabolites. The concentration of 17αOH-progesterone in adrenal venous blood was 8·6 times larger than its concentration in blood which was collected simultaneously from the femoral artery. This is evidence that it is actively secreted by the adrenal gland. The secretion of 17αOH-progesterone was affected by hypophysectomy and ACTH in the same way as the secretion of the glucocorticoids and the other precursor steroids.

In Table 2 observations on the concentration of 17αOH-progesterone in adrenal tissue of several species have been listed. In addition, pregnenolone, progesterone, the 17-oxo-steroids (androstenedione, adrenosterone and 11βOH-androstenedione) and the glucocorticoids were measured. From the first dog and the 10-week-old puppies adrenal blood had been collected for a period of 30 min before removal of the glands and the secretion rates of 17αOH-progesterone, the 17-oxo-steroids and the C-17, 21-hydroxy steroids are also included. The other animals were used as control animals in independent experiments on brain amines. Their adrenals were removed after rapid exsanguination under anaesthesia. In all three species the adrenal concentrations of 17αOH-progesterone, pregnenolone and progesterone were lower than the concentrations of the gluco- and mineralocorticoids.

DISCUSSION

The rate at which 17αOH-progesterone is secreted by the adrenal gland of the dog and its concentration in adrenal tissue was studied using a gas-chromatographic method. The amounts secreted were very small and it is doubtful whether they would be able to exert an effect on sodium excretion under normal conditions. The amounts are however in the same order of magnitude as those (250 mg/week) which were found to increase serum oxytocinase in pregnant patients (Semm, 1965).

The main significance of 17αOH-progesterone is the role which it plays as an intermediate compound in the synthetic pathway between progesterone and the C-17,21-hydroxy steroids and as a precursor of the 17-oxo-steroids which are formed in the adrenal gland. In previous experiments it was observed that the adrenal gland does not store any excess quantities of pregnenolone and progesterone, the first known C-21 precursors of the gluco- and mineralocorticoids (Holzbauer, 1968; Holzbauer & Newport, 1967). An increase in the rate at which pregnenolone is synthesized is therefore a prerequisite for any increase in the secretion of cortico-steroids. The present experiments have confirmed these observations and have in addition shown that the adrenal concentration of 17αOH-progesterone is also very low. From the results shown in Table 2,

TABLE 2. Adrenal concentration of 17αOH-pregn-4-ene-3,20-dione (17αOH-progesterone), 3βOH-pregn-5-en-20-one (pregnenolone), pregn-4-ene-3,20-dione (progesterone), the 17-oxo-steroids and the major glucocorticoids in several species

Species	Experimental conditions	Adrenal steroid concentrations (n-moles/g adrenal)						Secretion into adrenal venous blood (n-moles/g adrenal/min)		
		17αOH-progesterone	Pregnenolone	Progesterone	17-oxo* steroids	Compounds† $F+E+S$	Compounds† $A+B$	17αOH-progesterone	17-oxo-steroids	Compounds $F+E+S$
Dog, ♀ adult	Chloralose anaesthesia, adrenal blood collection	1·94	5·69	5·41	1·94	54·3	70·2	0·22	0·76	29·3
Puppies, mean of 2♂ and 1♀, 10 weeks	Chloralose or pentobarbitone anaesthesia, eviscerated and nephrectomized, adrenal blood collection	4·54	44·24	24·80	2·57	43·1	35·1	0·30	0·81	38·84
Dog, ♀ adult	Ether anaesthesia, acute exsanguination	3·03	10·74	18·13	1·79	56·2	48·4	—	—	—
Puppy, ♂ 3 months	Chloroform anaesthesia, acute exsanguination	4·24	17·06	4·13	—	145·8	201·1	—	—	—
Puppy, ♀ 3 months	Chloroform anaesthesia, acute exsanguination	9·38	28·74	9·22	—	257·4	188·6	—	—	—
Monkey, *macaca mulatta*, ♀ adult	Chloroform anaesthesia, acute exsanguination	16·04	11·69	6·04	—	52·9	21·4	—	—	—
Guinea-pigs, mean of 2♂, adult	Ether anaesthesia, acute exsanguination	9·08	37·92	25·44	—	130·2	—	—	—	—

* 17-oxo-steroids: sum of: androst-4-ene-3,17-dione (mol. wt. = 286·3), androst-4-ene-3,11,17-trione (mol. wt. = 300·3), 11βOH-androst-4-ene-3,17-dione (mol. wt. = 302·3).

† Chemical names as in legend of Table 1.

— Not analysed.

a quantitative comparison can be made between the steroids contained in the adrenal and the steroids secreted by the same tissue during a period of 30 min before the removal of the gland. It can be seen that the amounts of 17αOH-progesterone secreted in 1 min were equivalent to 11·3 and 6·6 % of the amounts of 17αOH-progesterone contained in the glands. In contrast, the amounts of the C-17,21-hydroxy steroids secreted in 1 min were equivalent to 54 and 90 % of the amounts contained in the glands. There was also no accumulation of 17-oxo-steroids in adrenal tissue. Thus, from the sum of the rates at which the 17-hydroxy steroids and the 17-oxo-steroids are secreted the approximate rate at which 17αOH-progesterone was synthesized can be calculated. In stressed dogs with intact pituitary glands it was found to be in the order of 30–40 n-moles/g adrenal/min. These calculations are necessarily based on three assumptions. First, hydroxylation is assumed to occur in position C-17 always before it occurs at C-11 or C-21; this is at present generally accepted. Secondly, it is assumed that the adrenal gland does not synthesize or secrete other steroids derived from 17αOH-progesterone which have so far escaped detection. Finally, it is assumed that there are no fundamental differences in the amounts of steroids retained in the adrenal glands *in situ* during the blood collection and after their removal from the body.

From the comparison of the rate at which the sum of the C-17,21-hydroxy steroids was secreted with the rate at which the sum of 17αOH-progesterone and the 17-oxo-steroids was secreted it can be seen that more than 97 % of the 17αOH-progesterone synthesized in the adrenal gland was hydroxylated in position C-21. The rate at which the C-21 hydroxylation took place can be assumed to be similar to the rate at which the C-17,21-hydroxy steroids were secreted and should therefore be 3 % slower than the rate at which 17αOH-progesterone was synthesized.

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ADRENAL SECRETION
RATES AND ADRENAL TISSUE CONCENTRATIONS OF PREGNENOLONE, PROGESTERONE, 11βOH-ANDROSTENEDIONE AND SOME OTHER STEROIDS IN YOUNG PIGS AND DOGS

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SUMMARY

1. The rates were measured at which a number of different steroids were secreted by the adrenal gland of the eviscerated and nephrectomized young pig and dog.

2. In addition to cortisol and corticosterone the following steroids were consistently found to be present in the adrenal venous blood: pregnenolone, progesterone, 11βOH-progesterone, androstenedione, 11βOH-androstenedione and adrenosterone.

3. The sum of the latter steroids could amount to as much as 30% of the total steroid secretion.

4. A severe deficit in the blood volume increased the secretion of pregnenolone and the 17-oxo steroids in the pig.

5. α-ethyltryptamine failed to inhibit ACTH release if the animals were eviscerated and nephrectomized, or if they were anaesthetized with chloralose instead of pentobarbitone sodium.

6. The same steroids as in the adrenal venous blood were found in extracts from the adrenal glands of a number of species.

7. The amount of individual steroids present in the adrenal gland of the stressed pig and dog was compared with the rate at which each had been secreted immediately before the excision of the gland.

8. There was a positive correlation between the adrenal concentrations and the secretion rates of cortisol, the major glucocorticoid secreted by the pig and the dog.

9. In contrast, the rate at which pregnenolone was secreted did not show a consistent relationship to its concentration in the gland.

10. A storage mechanism for pregnenolone in the adrenal cortex is proposed.

INTRODUCTION

The secretion of steroids from the adrenal cortex was first studied by Vogt (1943) in experiments in which the biological activity of adrenal venous blood of dogs was tested by its ability to prolong survival time. In the following years much information has been gathered on the formation and metabolism of individual steroids such as cortisol, corticosterone and aldosterone and on the factors which control their secretion. Most of the physiological functions of the adrenal cortex can be explained by the actions of these major gluco- and mineralocorticoids. There are, however, experimental and clinical observations which point to a possible biological role of other steroids secreted by the adrenal cortex and it appears of importance to obtain information on the adrenal secretion of steroids hitherto not extensively investigated. The presence in adrenal venous blood of small quantities of compounds which could be classified either as 'precursor steroids' or 'adrenal androgens' has been reported on several occasions (for references, see Dorfman & Ungar, 1965).

With the introduction of gas chromatographic methods it has become possible to measure nanogram quantities of certain types of steroids in blood and tissue extracts. Using these methods some quantitative observations were made on the secretion of pregnenolone, progesterone, 11βOH-progesterone and 11βOH-androstenedione by the adrenal gland of the dog, and its dependence on the pituitary gland was established (Heap, Holzbauer & Newport, 1966; Holzbauer & Newport, 1968*a*, *b*).

In the present experiments the adrenal secretion of these and some other steroids by the young pig and the puppy was studied. An attempt was made to correlate the secretion rates of steroids with the experimental conditions and with the concentrations of steroids in the adrenal gland.

METHODS

Operative procedures

Female pigs (Landrace or Large White), 8–12 weeks old, were used. They were anaesthetized with halothane, followed by chloralose. From pig No. 12 onwards phencyclidine HCl (Sernylan, Parke Davies and Co., 1-(1-phenylcyclohexyl) piperidine HCl, 1 mg/kg body weight) was injected subcutaneously before moving the animals from the pig-house to the laboratory. This caused sedation and the animals accepted the halothane without a struggle. It also reduced the required dose of chloralose from 70 to 40 mg/kg. Because of the peculiar anatomy of the adrenal region in the young pig, the animals had to be eviscerated and nephrectomized. The venous effluent of both adrenal glands was collected from a pocket formed from the inferior vena cava. The details of the operation have been described (Holzbauer & Newport, 1967*a*). Blood was collected over periods of 5–10 min.

The puppies, 10-week-old litter mates, were anaesthetized with halothane, followed either by chloralose (80 mg/kg I.V.) or by pentobarbitone sodium. In order to make the experimental conditions comparable to those of the pigs, they were also eviscerated and nephrecto-

mized and blood was collected from both adrenal glands via a caval pocket for periods of 30 min. The volume of blood removed was replaced by infusing into a femoral vein blood from an adult donor dog. Adrenal venous blood from adult dogs was collected as described by Holzbauer (1964). The adrenal glands from pigs and puppies were removed immediately after the last collection period.

The adrenal glands of five other species were also analysed for their steroid contents. Some of these animals had been used for other experiments prior to the excision of the glands. Details are included in the text.

The drugs used were α-ethyltryptamine acetate (Monase, Upjohn Co., Kalamazoo, U.S.A.), ACTH (Corticotrophin B.P., Organon Laboratories Ltd., for intravenous use, of porcine origin) and dextran (Dextraven, Benger Laboratories).

Chemical procedures

The following trivial names for steroids are used. Adrenosterone: androst-4-ene-3,11,17-trione; androstenedione: androst-4-ene-3,17-dione; compound A: 21-hydroxypregn-4-ene-3,11,20-trione; compound S: 17α,21-dihydroxypregn-4-ene-3,20-dione; corticosterone (compound B): 11β,21-dihydroxypregn-4-ene-3,20-dione; cortisol (compound F): 11β,17α,-21-trihydroxypregn-4-ene-3,20-dione; cortisone (compound E): 17α,21-dihydroxypregn-4-ene-3,11,20-trione; dehydroepiandrosterone (DHA): 3β-hydroxyandrost-5-en-17-one; DOC: 21-hydroxypregn-4-ene-3,20-dione; pregnenolone: 3β-hydroxypregn-5-en-20-one; progesterone: pregn-4-ene-3,20-dione; testosterone: 17β-hydroxyandrost-4-en-3-one.

The whole blood was diluted with 1 volume of water and extracted three times with 2 volumes of a mixture of ethylacetate and ether 2:1. The extracts were freed from fats and phospholipids (see Holzbauer, 1964). As for the estimation of glucocorticoids and aldosterone (Holzbauer & Vogt, 1961), the use of whole blood instead of plasma was desirable also for other steroids. In a study of the distribution of progesterone between plasma and blood cells in rat adrenal venous blood only one half of the total amount of progesterone present was obtained when the plasma fraction alone was extracted.

The adrenal glands were removed immediately after the last adrenal blood collection period or at the end of exsanguination. They were either stored at $-16°$ C or immediately homogenized in a mixture of ethylacetate and water, 2:1, in a glass homogenizer. The extracts were purified in the same manner as the blood extracts. Conjugated steroids were not extracted from either blood or glands by the procedures used.

In order to be able to correct for losses during the chemical procedures 4-[^{14}C]pregnenolone or 4-[^{14}C]progesterone was added to the whole blood before extraction and to the homogenates of the glands before centrifugation. However, these [^{14}C]labelled steroids had not been obtained at the time when the experiments on the pigs were done.

Descending paper chromatograms were developed at 29° C in the E_2B and E_1 system of Eberlein & Bongiovanni (1955), and the B_{5a}, the B_3 and the A system of Bush (Bush, 1952; Bush & Sandberg, 1953). For the B_{5a} and the E_2B system 3 cm lanes were used of Whatman No. 2 paper previously washed with the solvent mixture used for eluting the steroids off the paper (ethylacetate:methanol, 2:1). When the eluate of a paper chromatogram was to be examined by gas–liquid chromatography, the extracts had to be applied to NaOH-treated Whatman No. 50 paper (1·5 cm lanes); eluates from this paper produced only one small 'blank peak' and this was seen only if areas larger than 2 cm^2 were eluted (Sharman, 1963; Holzbauer & Newport, 1967*a*). The glucocorticoids in the adrenal blood extracts were estimated after chromatography of 1/20 of the total extract either in the E_2B or the B_{5a} system. For the estimation of the other steroids, the remainder of the blood extracts and the total adrenal gland extracts were first chromatographed in the E_2B system to free the samples from the bulk of the major glucocorticoids. The eluate of the region above corticosterone (including the solvent front) was rechromatographed in the E_1 or in the A system. This region contained compound S, DOC, the androstane derivatives and the pregnane

derivatives which do not have a hydroxyl group on C-21. From the second chromatogram the steroids present in the region of the first 5 cm, including the origin, were rechromatographed in the B_3 system for 8 hr. Individual steroids were eluted from the final chromatograms according to the position of marker substances and U.V. absorption. Aldosterone was separated as described by Holzbauer & Vogt (1961).

Gas–liquid chromatography was carried out on an F & M Model 402 gas chromatograph fitted with a flame ionization detector maintained at 260° C. A 3·8 % SE-30 column (length 122 cm, oven temperature 230° C) was used. The carrier gas was argon. Retention times were calculated in relation to that of cholestane (about 20 min) and expressed as relative retention times (RRT). A few substances were also applied to an XE-60 chromatography column to aid in their identification. On this column the retention times were expressed relative to that of cholesterol. Those steroids which were stable under these conditions and which had on the 3·8 % SE-30 column a retention time shorter than that of cholestane could usually be detected in quantities of about 10 ng, corresponding to 0·05 μg per eluate. When they were retained for a longer time the peaks became wider and increasing quantities were required.

Quantitative estimation of steroids. Those steroids which have a hydroxyl group in position C-21 were estimated by their colour reaction with blue tetrazolium (Vogt, 1955). If present in quantities of less than 2 μg a modification of this method (Holzbauer & Vogt, 1961) was used. In the B_{5a} system, compounds B, A and S do not become separated if present in larger quantities and were eluted together. Thus the estimates for corticosterone include also compounds A and S. The chromatographic systems will be indicated in the tables.

All other steroids were estimated by gas chromatography (Heap *et al.* 1966; Holzbauer & Newport, 1967*b*, 1968*b*). These methods are based on the straight-line relationship which exists between the concentration ratio of two steroids in a solution and the ratio of the heights of their peaks shown on the gas chromatograph tracing. The reference substance used for the estimation of androstenedione, pregnenolone and progesterone was 11βOH-progesterone; for the estimation of 11βOH-androstenedione the reference was pregnenolone; for the estimation of 11βOH-progesterone and of adrenosterone, androstenedione was used. 11βOH-androstenedione, 11βOH-progesterone and adrenosterone were usually estimated in eluates of the corresponding regions in the B_3 chromatograms; androstenedione, pregnenolone and progesterone in eluates from the A or E_1 chromatogram. In those experiments in which [^{14}C]pregnenolone and [^{14}C]progesterone were added a portion of the eluates was used for counting the remaining radioactivity in a Tricarb scintillation spectrometer.

Formation of derivatives. Oxidation with chromium trioxide (CrO_3), reduction with sodium borohydride ($NaBH_4$) and acetylation were carried out as described previously (Holzbauer & Newport, 1968*b*), and enzymic hydroxylation in position C-20 according to Heap (1964).

Chemicals. All solvents used were purified as described by Holzbauer & Vogt (1961) and by Heap *et al.* (1966). Reference steroids were obtained from Professor W. Klyne (M.R.C. Steroid Reference Collection).

RESULTS

Chemical nature of steroids extracted from adrenal venous blood and glands

Tests for the chemical nature of steroids were carried out either on extracts of a large volume of blood collected from one pig or dog or on pooled eluates of appropriate regions of paper chromatograms on which adrenal blood or gland extracts of members of the same species had been developed. The identifications were based mainly on comparing the behaviour of an unknown compound with that of an authentic steroid in

several paper and gas chromatographic systems and on the behaviour of their reaction products after reduction, oxidation, acetylation and enzymic hydroxylation. In the case of pregnenolone a mass spectrogram was obtained (Holzbauer & Newport, 1968*a*). Evidence for the chemical identity of 16αOH-progesterone (Holzbauer & Newport, 1967*a*), 17αOH-progesterone (Holzbauer & Newport, 1968*b*), progesterone, 11βOH-progesterone and 11βOH-androstenedione (Heap *et al.* 1966) in adrenal blood and gland extracts has been presented in previous papers. These observations were further substantiated for 11βOH-androstenedione by reducing the 'unknown' compound with $NaBH_4$ to a compound behaving like 11βOH-testosterone (RRT 0·92, SE-30 column of the gas chromatograph), and for 11βOH-progesterone by the formation of 11-ketoprogesterone (RRT 1·07, SE-30 column) on oxidation with CrO_3.

Identification of androstenedione in the extract of 1 l. adrenal venous blood (collected from a young male dog over a period of 5 hr) was based on its Rf value in the E_2B, B_3, E_1 and A systems, its RRT on the gas chromatograph (0·48, SE-30 column; 1·28, XE-60 column), and its transformation to testosterone by reduction with $NaBH_4$.

From the same blood extract a compound was isolated which behaved similarly to adrenosterone on the paper chromatograms and on the SE-30 column of the gas chromatograph (RRT 0·59). After reduction with $NaBH_4$ a compound with the properties of 11-ketotestosterone (RRT 0·72, SE-30 column) was obtained.

There were also small amounts of a compound present in the blood extract which travelled originally like 11-ketoprogesterone. Like the reference standard, it could be converted enzymically into a substance moving like 20βOH-pregn-4-ene-3,11-dione (RRT 1·22, SE-30 column).

In addition, this extract contained small amounts of a substance which had several characteristics in common with 17αOH-pregnenolone. They were: its R_F value in the E_2B system was similar to that of compound S, in the B_3 system it remained on the origin, even if the chromatogram was developed for 24 hr. When injected on to a 3·8 % SE-30 column it produced a peak at RRT 0·84 with a shoulder at RRT 1·08. The RRT of this double peak changed after acetylation to RRT 1·27 with a shoulder at RRT 1·59. Any attempt to find this steroid in adrenal blood of puppies (pooled sample of 580 ml., 2 hr collection) or of pigs (pooled sample of 800 ml., 2 hr. collection) failed.

A substance with the same RRTs as 17αOH-pregnenolone, and, after acetylation, as its acetate, was found on the B_3 chromatogram of many adrenal blood extracts in the region situated between 11βOH-androstenedione and 11βOH-progesterone. Its separation from 11βOH-androstenedione (RRT 0·84) was sometimes incomplete. Since 11βOH-androstenedione

does not form an acetate under the conditions employed, eluates of this region were acetylated after estimation of the '11βOH-androstenedione' on the gas chromatograph. When the eluates were contaminated by the unknown substance a peak at 1·27 appeared and corresponding corrections of the estimate could be made.

Adrenal secretion of steroids in the young pig and the puppy

The rates at which thirteen different steroids were secreted by the adrenal gland of the young pig are listed in Table 1. With the exception of pig No. 2, all animals were eviscerated and bilaterally nephrectomized. Adrenal blood collection was started shortly after completion of surgery. Only the figures for aldosterone were corrected for losses. In subsequent experiments in which recovery was checked by the addition of [^{14}C]-labelled pregnenolone and [^{14}C]progesterone to the blood samples, the mean recovery from volumes ranging from 30–200 ml. was 40 %. In addition to the major glucocorticoids and aldosterone, the adrenal venous blood of the piglets contained substantial quantities of steroids which are either intermediate compounds in the synthetic pathway leading to the major adrenal steroids or 17-oxo steroids with androgenic activity. In samples of arterial blood of the same piglets these steroids were either not detectable or their concentrations were 10–20 times lower than in the adrenal venous blood. This indicates that the steroids listed in Table 1 were actively secreted by the adrenal cortex.

The secretion rates of all steroids varied widely between individual animals. The percentage contributed by each steroid to the total steroid secretion was also variable. There was no correlation between the amount of precursor steroids secreted and the total steroid secretion. The secretion of pregnenolone and the sum of the 17-oxo steroids (androstenedione + adrenosterone + 11βOH-androstenedione) ran parallel in individual animals. In Fig. 1 the secretion rates of pregnenolone are plotted against the rates at which the 17-oxo steroids were secreted. Observations made previously on a group of six adult dogs are included. This correlation could indicate that the secretion of these steroids is influenced by a common factor. Progesterone and 11βOH-progesterone were also secreted at fairly similar rates in individual animals.

From some of the pigs listed in Table 1 more than one adrenal blood sample was collected. The experiment on pig No. 6 provided the possibility to study the effects which changes of the blood volume, the arterial pressure and the loss of red blood cells exerted on the secretion of six different steroids. The observations are summarized in Fig. 2. Adrenal venous blood was collected over consecutive periods of 10 min. During the first 25 min the volume of the blood withdrawn was not replaced and

TABLE 1. Secretion of steroids by the adrenal gland of the piglet

Anaesthesia was induced with halothane and maintained with chloralose. Pigs 13, 14 and 15 were premedicated with Sernylan. With the exception of pig No. 2 all animals were eviscerated and nephrectomized. Adrenal blood was collected from both glands, in pig No. 2 from the left gland only. Collection started immediately after completion of surgery. L.R.: Landrace; L.W.: Large White; L.M.: litter mates; —: not analysed; n.d.: not detected; adr. wt.: weight of adrenal glands; coll.: adrenal blood collection; *chromatography in the B_{5a} system, estimate includes compounds A and S. No correction for losses was made.

Pig no. and sex	Race	Body wt. (kg)	Age (weeks)	Adr. wt. (mg)	Coll. time (min)	Mean B.P. (mm Hg)	Steroid secretion rates (n-mole/g adrenal/min)													
							Compound													
							F	B	E	A	S	Pregnenolone	Progesterone	11βOH-progesterone	DOC	Aldosterone	Androstenedione	11βOH-androstenedione	Adrenosterone
2	♂ L.R.	14	7	0·60	45	115	39·2	18·0*	—	—	—	—	—	—	—	—	—	—	—
6	♀ L.W.	12	9	1·73	10	50	44·8	41·8*	—	—	—	2·03	0·88	0·71	3·13	1·19	n.d.	0·96	n.d.
L.M. 7	♀ L.R.	19	10	1·90	10	—	16·2	12·9	0·56	1·97	3·26	—	—	—	—	1·21	—	—	—
L.M. 8	♀ L.R.	21	10	2·40	10	65	32·6	21·5*	—	—	—	1·14	0·49	2·04	—	1·18	—	1·40	0·21
9	♀ L.R.	21	12	1·95	10	—	24·3	13·6*	—	—	—	5·86	1·82	3·28	—	—	1·22	4·80	0·31
13	♀ L.R.	20	14	2·47	10	85	79·5	32·3*	—	—	—	3·26	7·15	7·74	—	—	0·25	1·48	0·03
L.M. 14	♀ L.W.	18	12	1·68	5	85	14·6	13·4	2·72	1·22	6·41	2·32	4·58	4·27	—	—	1·15	1·09	0·22
L.M. 15	♀ L.W.	20	12	2·36	10	62	16·3	4·0	0·83	0·81	2·37	0·53	0·50	0·36	0·58	—	0·12	0·59	0·04

the animal lost at least 140 ml. blood. Consequently, arterial blood pressure and adrenal blood flow decreased. This was accompanied by a fall to less than one half in the secretion rates of cortisol and corticosterone (▲), aldosterone (▷), progesterone (●) and 11βOH-progesterone (○). In contrast, there was a steep increase in the rate at which 11βOH-androstenedione (×) and pregnenolone (△) were secreted. During the second half of the third collection period an I.V. infusion of dextran was started. This

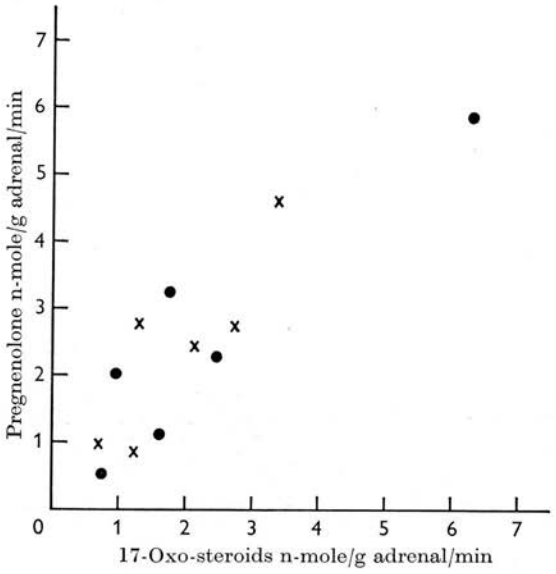


Fig. 1. Correlation between the secretion rates of pregnenolone and the 17-oxo steroids (androstenedione + 11βOH-androstenedione + adrenosterone) in the young pig (●) and the dog (×).

caused a rise of the arterial blood pressure which reached a maximum during the sixth collection period. The increase in the blood pressure restored the secretion rates of those steroids the secretion of which had been depressed. Later in the experiment the haematocrit became very low and the secretion rates fell again in spite of adequate blood pressure, probably due to the impaired oxygen supply.

The response of 11βOH-androstenedione and pregnenolone secretion to the restoration of the blood volume was somewhat slower, a fall becoming apparent only in the fifth collection period. During the second half of the experiment the volume of blood withdrawn was not fully replaced and the calculated blood volume fell again. This was followed by a slight rise in the secretion of pregnenolone and 11βOH-androstenedione in sample No. 7. However, during the eighth collection period haematocrit

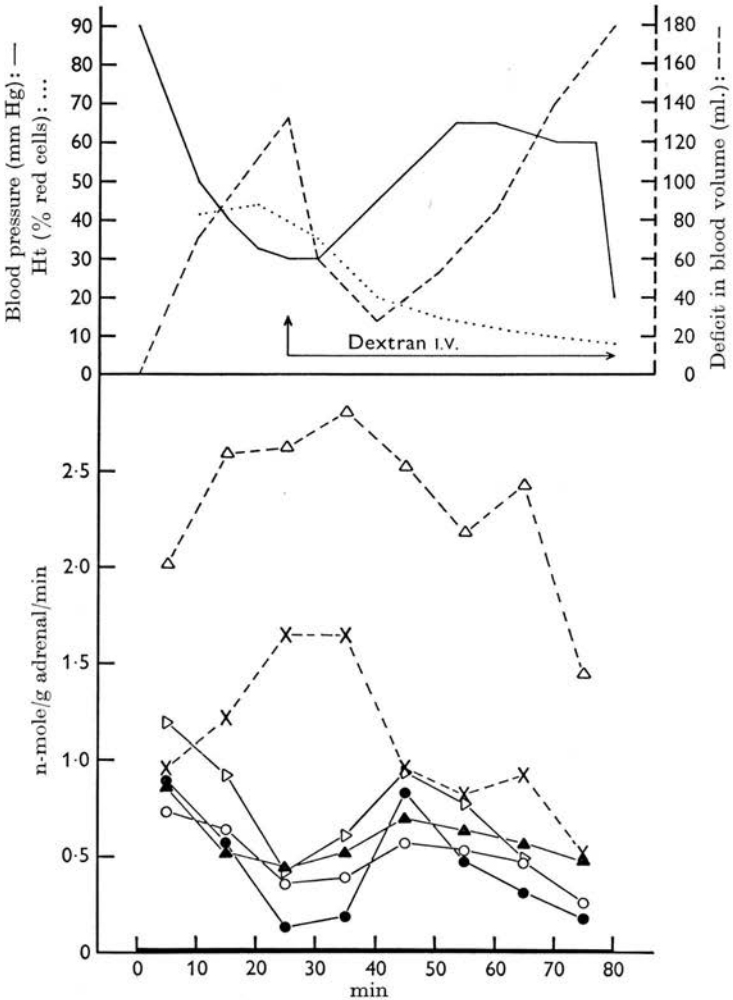


Fig. 2. Adrenal steroid secretion in pig No. 6 (♀, 9 weeks, 13 kg), chloralose anaesthesia; eviscerated. Time 0: 3 hr after induction of anaesthesia with halothane, start of adrenal blood collection; eight samples were collected consecutively over periods of 10 min. Upper diagram: — = blood pressure; ···· = haematocrit (Ht); - - - = deficit in blood volume; at arrow infusion of dextran into femoral vein started. Lower diagram: steroid secretion rates: △---△ = pregnenolone; ×---× = 11βOH-androstenedione; ▷—▷ = aldosterone; ○—○ = 11βOH-progesterone; ●—● = progesterone; ▲—▲ = cortisol + corticosterone. The figures for cortisol and corticosterone were divided by 100. No corrections for losses were made.

and blood pressure were too low to maintain the normal rate of synthesis of any of the adrenal steroids.

In Fig. 3, the secretion rates of pregnenolone and of the sum of the 17-oxo steroids (androstenedione, 11βOH-androstenedione and adreno-

sterone) are plotted against the deficit in blood volume calculated by subtracting the amount of donor blood infused from the amount of adrenal blood collected. The observations were made on five pigs during the first 10 min collection period of adrenal blood. It can be seen that the highest pregnenolone and 17-oxo steroid secretion rates coincided with the most severe deficit in reinfusion and vice versa. There was no correlation between the secretion rates of pregnenolone or 17-oxo steroids and the adrenal blood flow. The highest blood flow was seen in the two animals with the lowest pregnenolone secretion.

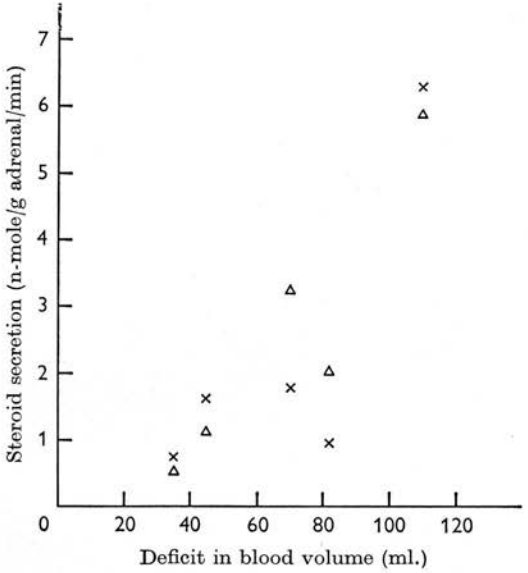


Fig. 3. Correlation of adrenal secretion rates of pregnenolone and of 17-oxo steroids (sum of androstenedione, 11βOH-androstenedione and adrenosterone) with the volume of blood withdrawn and not replaced by donor blood. Experiments on piglets Nos. 6, 8, 9, 13 and 15. All data were from the initial adrenal blood collection period of 10 min. △: pregnenolone, × : 17-oxo steroids.

Table 2 summarizes observations made on a litter of three 10-week-old puppies. In order to obtain experimental conditions comparable to those of the experiments on the pigs, the puppies were eviscerated and nephrectomized and blood was collected from a caval pocket. At the time of these experiments radioactive pregnenolone and progesterone had been obtained and the figures in Table 2 are corrected for losses. From puppy No. 2, arterial blood (40 ml.) was collected at the end of the adrenal blood collection. Whereas the concentration in adrenal blood of the precursor steroids ranged from 27 to 140 ng/ml. and of the 17-oxo steroids from 3 to 31 ng/ml., none of these steroids could be detected in the arterial blood, i.e. their

TABLE 2. Secretion of steroids by the adrenal gland of the puppy

The puppies (litter mates, 10 weeks old) were eviscerated and nephrectomized. Adrenal blood was collected for 30 min from both glands. Collection was started 5 min after completion of surgery. The blood withdrawn was replaced by donor blood. The figures for pregnenolone and progesterone were corrected for losses. n.d.: not detected.

Puppy no. and sex	Body wt. (kg)	Adrenal wt. (g)	Anaesthetic	Mean B.P. (mm Hg)	Blood coll. (ml.)	Steroid secretion rates (n-mole/g adrenal/min)											
						Compound											
						F	B	A	E	S	Pregnenolone	Progesterone	11βOH-progesterone	DOC	Androstenedione	11βOH-androstenedione	Adrenosterone
1 ♂	3	0·46	Chloralose (80 mg/kg)	140	120	33·9	19·0	5·3	2·4	1·9	3·44	0·97	1·05	0·38	0·12	0·24	n.d.
2 ♀	3	0·44	Pentobarbitone sodium (40 mg/kg)	130	55	27·3	12·3	3·8	1·7	2·2	1·85	0·46	0·35	0·46	0·06	0·42	0·04
3 ♀	3	0·42	Pentobarbitone sodium (40 mg/kg)	148	154	38·2	23·4	3·7	2·6	3·9	4·52	1·10	0·96	0·91	n.d.	0·43	0·07

concentration in arterial blood was less than 2 ng/ml. This observation confirms that they are actively secreted by the adrenal gland. There was no obvious difference between the steroid secretion rates of the two puppies anaesthetized with pentobarbitone sodium and the puppy anaesthetized with chloralose. Calculated per gram adrenal gland, the secretion of the glucocorticoids, DOC and pregnenolone was similar in piglets and puppies. Secretion of progesterone and 11βOH-progesterone was lower in the puppies, even after correcting the figures for losses.

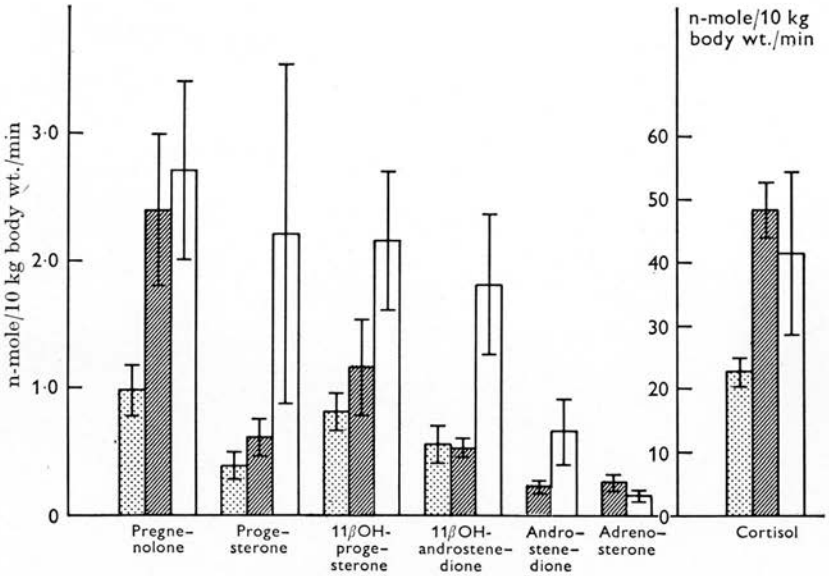


Fig. 4. Comparison of steroid secretion rates/kg body wt. of dogs (▦), puppies (▨) and young pigs (□). All animals were under operative stress, the puppies and pigs also eviscerated and nephrectomized. The figures were not corrected for losses; the standard errors of the means are indicated.

In order to be able to compare the amount of steroids available per kg body weight in the two species, the secretion rates were also calculated in moles/min/kg body weight (previous to evisceration). The figures used were not corrected for losses. The results are shown in Fig. 4. It includes observations made earlier on a group of six adult, not nephrectomized or eviscerated dogs. The individual variations were largest in the pigs, probably due to the more complicated operation. The secretion rates of cortisol and pregnenolone were higher in puppies and piglets than the adult dogs. There was no difference in the secretion rates of progesterone, 11βOH-progesterone and 11βOH-androstenedione between adult dogs and puppies. In the piglets the secretion rates of these three steroids were higher. The values for corticosterone are not shown on the diagram because

in most of the dog experiments they were obtained after chromatography in the B_{5a} system and include also compounds A and S.

Effect of α-ethyltryptamine on adrenal steroid secretion

In previous experiments on hypophysectomized dogs it was observed that the adrenal secretion of 17-oxo steroids and of those pregnane derivatives which are not hydroxylated at C-21 is dependent on the presence of the pituitary gland (Heap *et al.* 1966). After hypophysectomy the secretion rates of all steroids measured were decreased to a similar extent but secretion did not cease completely. Infusion of ACTH restored the secretion of all steroids to prehypophysectomy rates (Holzbauer & Newport, 1968*a*, *b*). The question arose whether some other pituitary polypeptides might have a more specific effect on the secretion of an individual steroid. For this type of experiment, the pig seemed to be more suitable than the dog, because it is possible to obtain porcine pituitary polypeptides. Unfortunately, in the pig hypophysectomy is a much more difficult operation than in the dog, and, after the involved surgery required for adrenal blood collection, the final preparation would be far removed from physiological conditions. An attempt was therefore made to achieve 'chemical hypophysectomy' with the help of α-ethyltryptamine. This substance was found by Tullner & Hertz (1964) and by Ganong, Wise, Shackleford, Boryczka & Zipf (1965) to decrease the release of corticotrophin (ACTH) by inhibiting the release of the corticotrophin-releasing factor in dogs anaesthetized with pentobarbitone sodium.

Experiments were carried out on two pigs which were anaesthetized with chloralose, eviscerated and nephrectomized (Figs. 5 and 6, Table 3). A first adrenal blood sample was collected for a period of 10 min. Then a 0·2 % solution of α-ethyltryptamine in 0·9 % sodium chloride was infused intravenously. Both animals responded with a blood pressure fall instead of the rise seen in the dog. In the first experiment (Fig. 5) pig's blood was used to replace the volume of blood withdrawn and the haematocrit remained unchanged. The mean blood pressure during the first collection period was 65, during the second and third 50 mm Hg. ACTH was infused at a rate of 0·45 m-u./min/kg initial body wt. In the second experiment (Fig. 6) the blood volume was kept constant by infusing dextran, which caused a fall in the haematocrit. The blood pressure before α-ethyltryptamine was 62 mm Hg. Afterwards it was kept constant at about 55 mm Hg until the last collection period when it fell to 30 mm Hg within 5 min. ACTH was infused at 10 times the rate of the previous experiment. In both pigs α-ethyltryptamine had but little effect on the 'stress' secretion of the major glucocorticoids and, not surprisingly, ACTH infusion was without effect. In the second experiment another three adrenal blood

samples were collected and a continuous fall in the secretion rates of all steroids parallel to the fall in the haematocrit was seen.

The failure of these pigs to respond to α-ethyltryptamine with a decrease in adrenal steroid secretion could have any of at least three causes. First, the difference in the species, second, the different anaesthetic, and third the additional operations of evisceration and nephrectomy.

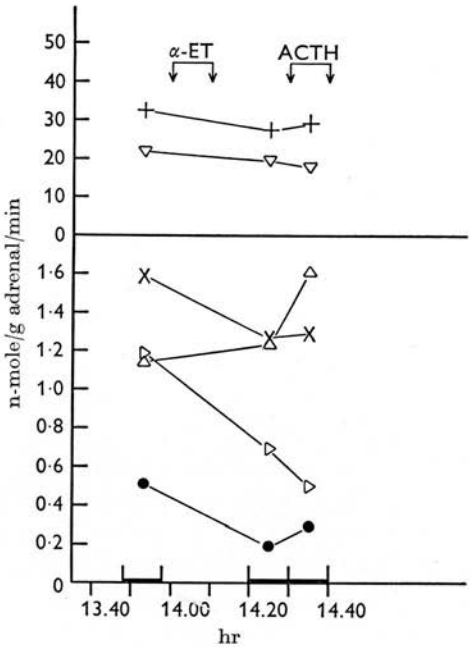


Fig. 5. Adrenal steroid secretion in pig No. 8 (♀, 10 weeks, 21 kg). Anaesthesia induced with halothane 10.00 a.m., followed by chloralose, eviscerated, nephrectomized. α-ET: α-ethyltryptamine: I.V. 0·2 % solution, 9·5 mg/kg body wt. ACTH: 0·43 m-u./min/kg body wt. Blood lost replaced by infusing pig's blood. Dark bars on abscissa: adrenal blood collection periods; +: cortisol, ▽: corticosterone; ×: 17-oxo steroids; △: pregnenolone; ▷: aldosterone; ●: progesterone. Figures not corrected for losses.

To investigate these points some experiments were carried out on dogs. The observations are summarized in Table 3.

When an adult dog (No. 2) was anaesthetized with pentobarbitone sodium and the left adrenal vein was cannulated via a flank incision, adrenal secretion of the major glucocorticoids was decreased by more than 90 % after an infusion of α-ethyltryptamine. The same dose of α-ethyltryptamine in a dog (No. 1) operated in the same way but anaesthetized with chloralose did not have any effect on steroid secretion. α-ethyltryptamine failed also to depress steroid secretion in a puppy (No. 3)

TABLE 3. α-Ethyltryptamine and adrenal steroid secretion

Adrenal blood was collected before and after an intravenous infusion of a 0·2 % solution of α-ethyltryptamine (α-ET) in 0·9 % sodium chloride. In the two adult dogs two or three samples were collected at 5 min intervals before α-ET. Blood collection after α-ET was started in the two adult dogs 15 min and in the pigs and the puppy 25 min after the beginning of the drug infusion. Further samples were taken at intervals of 15 min. The secretion rates of the major glucocorticoids were used as an index of ACTH release. (M: mean secretion during 2 or 3 collection periods; S: adrenal blood sample; evisc.: eviscerated; nephr.: nephrectomized; a.v. can.: adrenal vein cannulation; diss.: dissection.)

					α-ethyltryptamine infusion (I.V.)			Adrenal blood collection			Adrenal secretion rates of compounds F+B+S	
Species	Body wt. (kg)	Operative procedures	Anaesthetic	B.P. at end of diss. (mm Hg)	Time (min)	Dose (mg/kg)	Change in B.P. (mm Hg)	Conditions	Coll. time (min)	B.P. (mm Hg)	n-mole/g adrenal/min	% change after α-ET
Piglet No. 8 ♀	21	evisc. nephr. a.v. can.	Chloralose (70 mg/kg)	70	10	9·5	−20	S_1: before α-ET	10	65	54·1	−13
								S_2: after α-ET	10	50	47·3	
Piglet No. 15 ♀	20	evisc. nephr. a.v. can.	Sernylan + chloralose (40 mg/kg)	80	25	6·0	−20	S_1: before α-ET	10	62	22·7	−13
								S_2: after α-ET	8	58	19·8	
Dog No. 1 ♀	11		Chloralose (65 mg/kg)	150	4	10·0	+30	S_1, S_2: before α-ET	2×3	155	M: 49·8	− 5
								S_3, S_4: after α-ET	2×3	165	M: 47·3	
Dog No. 2 ♀	11	a.v. can.	Pentobarbitone sodium (29 mg/kg)	130	4	9·5	+80	S_1:S_3: before α-ET	3×3	140	M: 42·1	−93
								S_4-S_6: after α-ET	3×3	170	M: 2·9	
Puppy No. 3 ♀	3	evisc. nephr. a.v. can.	Pentobarbitone sodium (40 mg/kg)	148	6	10·0	+40	S_1: before α-ET	30	148	65·5	+ 1
								S_2: after α-ET	30	155	66·0	

anaesthetized with pentobarbitone sodium but eviscerated and nephrectomized. Thus, the lack of effect in the piglet could be ascribed to both the use of chloralose and the additional operation of evisceration and nephrectomy.

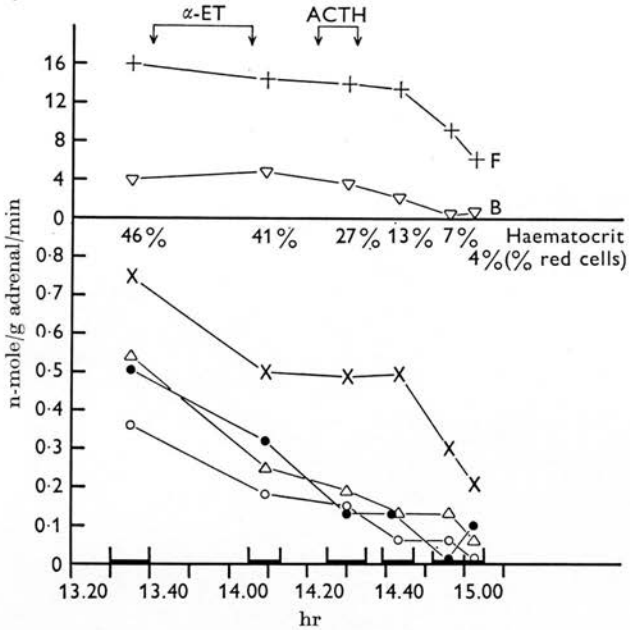


Fig. 6. Adrenal steroid secretion in pig No. 15 (♀, 12 weeks, 20 kg). Anaesthesia induced with halothane 9.40 a.m., followed by chloralose; eviscerated, nephrectomized. α-ET: α-ethyltryptamine: I.V. 0·2% solution, 6 mg/kg body wt.; 200 ml. pig's blood infused between 13·33 and 14·13. ACTH: I.V. 4·5 m-u./min/kg body wt. 900 ml. dextran infused between 14·15 and 15·03. Dark bars on abscissa: adrenal blood collection periods. +: cortisol; ▽: corticosterone; ×: 17-oxo steroids; △: pregnenolone; ●: progesterone; ○: 11βOH-progesterone. Haematocrit = % red cells. Figures not corrected for losses.

Steroid concentrations in adrenal tissue

The mean concentrations of a number of steroids in the adrenal glands of different species are listed in Table 4. All animals had been exposed to stressful stimuli before the adrenal glands were removed. The pig and dogs were either exsanguinated under ether or halothane anaesthesia or had been used for adrenal blood collection. The cats and monkeys were used in different experiments on brain amines and some of them had been treated with chlorpromazine, thioridazine or tranylcypromine. Neither drug seemed to have a consistent effect on adrenal steroid concentrations and the results were pooled. Cats and monkeys were exsanguinated under chloroform anaesthesia. The observations on rats were made on a group

TABLE 4. Mean steroid concentration in the adrenals of different species after stress

Figures not corrected for losses. Mean ± standard error of the mean; n.d.: not detected; —: not analysed

	Steroid concentration (n-mole/g adrenal)											
	Compound											
Species	F	B	A	E	S	Pregnenolone	Progesterone	11βOH-progesterone	DOC	Androstenedione	11βOH-androstenedione	Adrenosterone
Pig (n = 5)	41 ± 10·3	39 ± 5·6	—	—	—	36·0 ± 7·7	30·1 ± 4·7	4·4 ± 1·6	—	< 0·3	5·6 ± 1·6	0·4 ± 0·08
Dog (n = 5)	34 ± 4·8	29 ± 4·2	16 ± 4	10 ± 3·0	4 ± 1·7	9·3 ± 3·5	8·1 ± 2·1	1·6 ± 0·1	1·0 ± 0·34	7·5, 0·6	0·9 ± 0·2	0·2 ± 0·05
Cat (n = 8)	29 ± 4·5	26 ± 4·4	32 ± 4·4	13 ± 1·5	9 ± 0·7	4·3 ± 1·4	3·7 ± 0·9	n.d.	6·2 ± 0·5	0·7 ± 0·1	1·7 (n = 1)	n.d.
Monkey (*Macaca mulatta*) (n = 4)	29 ± 1·3	20 ± 4·2	19 ± 4·5	14 ± 2·4	8 ± 0·2	1·1 ± 0·7	1·7 ± 0·1	n.d.	4·4 (n = 2)	0·7 (n = 1)	n.d.	n.d.
Rat (n = 12)	n.d.	161 ± 0·7	—	—	—	24·5 ± 2·7	27·4 ± 3·1	n.d.	—	n.d.	n.d.	n.d.
Soay sheep (n = 1)	42	11	—	—	—	5·3	7·6	n.d.	—	0·4	0·6	n.d.
Horse (n = 1)	30	43	34	24	27	6·1	2·4	n.d.	—	n.d.	n.d	n.d.

TABLE 5. Concentration of steroids in the adrenal gland of the dog and the puppy

Pregnenolone and progesterone figures corrected for losses; n.d.: not detected; —: not analysed; *: secretion rates of compounds F, B and S decreased after α-ethyltryptamine; evisc.: evisceration; nephr.: nephrectomy

Animal no. and sex	Anaesthesia	Experiment	Adrenal tissue analysed (g)	Steroid concentration (n-mole/g adrenal)											
				Compound					Pregnenolone	Progesterone	11βOH-progesterone	DOC	Androstenedione	11βOH-androstenedione	Adrenosterone
				F	B	A	E	S							
Adult dogs:															
1 ♀	Chloralose	Adrenal blood collection; α-ethyltryptamine, 60 min before removal of gland	1·54	19·8	18·0	—	—	—	78·7	20·8	1·77	—	7·5	1·12	—
*2 ♀	Pentobarbitone sodium		1·42	2·7	3·7	—	—	—	30·6	6·0	n.d.	—	—	—	—
3 ♀	Chloralose		1·12	—	—	—	—	—	59·7	39·0	—	—	—	—	—
4 ♂	Ether	Exsanguinated	1·64	21·4	27·9	10·6	9·0	6·6	16·7	15·1	2·0	2·0	0·6	0·7	0·24
5 ♂	Halothane	Exsanguinated	1·51	46·6	34·0	14·4	8·0	1·5	10·8	18·3	1·5	2·1	n.d.	0·6	0·07
6 ♂	Chloralose	Adrenal blood collection (300 min)	1·55	45·2	27·3	—	5·2	—	6·1	25·5	n.d.	—	n.d.	1·26	n.d.
Puppies: (litter, 10 weeks)															
1 ♂	Chloralose	Evisc. nephr. adrenal blood collection (30 min)	0·46	25·2	21·3	12·6	12·0	5·3	33·5	33·7	2·0	0·7	n.d.	1·0	0·4
2 ♀	Pentobarbitone sodium		0·44	19·5	21·1	12·6	12·0	8·2	24·0	18·7	n.d.	0·7	0·5	1·4	0·4
3 ♀	Pentobarbitone sodium	Evisc. nephr. adrenal blood coll. 70 min; α-ethyltryptamine 40 min before removal of gland	0·42	28·3	26·2	11·4	10·6	8·0	75·0	30·7	1·6	1·7	n.d.	1·6	0·4

TABLE 6. Concentration of steroids in the adrenal gland of the piglet

Piglets, ♀, 8–12 weeks, 15–23 kg; Nos. 7, 9. 10, 14, 15: halothane anaesthesia, followed by chloralose, eviscerated, nephrectomized. *: chromatography in the B_{5a} system, estimate includes compounds A and S. L.M. = litter mate; Ht = haematocrit: adr. bl. = adrenal venous blood. Figures not corrected for losses. Pig No. 15: blood withdrawn replaced by dextran, α-ethyltryptamine 60 min, ACTH 25 min before removal of glands (see Fig. 6)

Pig no. and sex	Experimental conditions	Duration of experiment (min)	Steroid concentration (n-mole/g adrenal)						
			Compound		Pregnenolone	Progesterone	11βOH-progesterone	Androstenedione	11βOH-androstenedione	Adrenosterone
			F	B*						
7 ♀	1 adr. bl. sample coll. (10 min) exsanguinated	200	34·8	41·9	30·3	18·8	0·3	0·3	2·7	0·3
9 ♀	1 adr. bl. sample coll. (10 min) exsanguinated	210	44·3	59·5	38·7	28·2	3·3	0·3	2·7	0·3
10 ♀	1 adr. bl. sample coll. (1 min) heart failure	240	21·5	30·1	21·8	22·9	2·4	0·3	9·6	0·3
14 ♀ L.M.	1 adr. bl. sample coll. (5 min) exsanguinated	210	24·8	35·8	64·8	35·0	6·4	0·3	3·6	0·7
15 ♀ L.M.	6 adr. bl. samples coll. Ht = 4·1% at end of experiment	325	6·3	18·2	41·4	7·6	0·3	1·5	1·0	0·3
11 ♀	Ether anaesthesia, exsanguinated only	30	78·9	28·6	24·6	45·5	9·6	0·3	9·4	0·5

which were bled under ether anaesthesia. The sheep had been subjected to cranial surgery and the horse was exsanguinated after it had been given a large dose of chloral hydrate. Because [^{14}C]progesterone and [^{14}C]pregnenolone were only added in a few experiments, none of the figures shown in Table 4 were corrected for losses.

The steroids found in adrenal venous blood were also detected in the adrenal gland extracts. The relative concentrations of pregnenolone and progesterone were higher in adrenal tissue than in adrenal venous blood, especially in the pig and the rat. Those of 11βOH-progesterone and of the 17-oxo steroids were much lower. The large standard errors indicate the large individual variations. Compound A was the predominant C-21 hydroxysteroid in a number of cat adrenals.

The concentrations of steroids in the adrenal glands of individual dogs and puppies are listed in Table 5, of young pigs in Table 6. In Table 5 the figures for pregnenolone and progesterone are corrected for losses. The experimental conditions prior to the removal of the glands are indicated in the tables. The concentrations of steroids in the adrenal tissue varied between the individual animals. The adrenal gland of dog No. 2 showed the lowest steroid concentrations, with the exception of that of pregnenolone. In this dog secretion of steroids had been decreased by an I.V. injection of α-ethyltryptamine (see Table 3). Among the pigs the steroid concentrations in the adrenal were lowest in pig No. 15 in which secretion of steroids was also very low at the end of the experiment (see Fig. 6). However, as in dog No. 2, the pregnenolone concentration in the adrenal gland of pig No. 15 was within the normal range. The highest pregnenolone concentrations were found in the adrenals of dogs Nos. 1 and 3 and of puppy No. 3. To these animals α-ethyltryptamine had been given, but failed to cause a decrease in steroid secretion rates (see Table 3).

Relationship between the concentration of steroids in the adrenal gland and the rate of their secretion

The rates at which steroids had been secreted prior to the removal of the adrenal gland were measured in the experiments on the three puppies (see Table 2), on dogs Nos. 1 and 2 and on pigs Nos. 9, 14 (see Table 1) and 15 (see Fig. 6). Thus it was possible to compare directly the concentration of a steroid in the adrenal gland and its secretion rate; the rates during the last collection period were used for this comparison.

Figure 7 shows the correlation between the adrenal concentrations and the secretion rates of cortisol, the major glucocorticoid of the dog and the pig.

The relationship between adrenal concentrations and adrenal secretion rates of a number of individual steroids is illustrated in Figs. 8–10. Figure 8

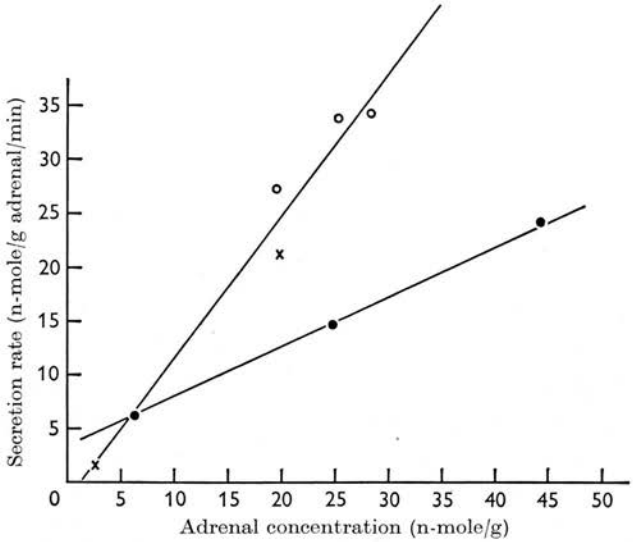


Fig. 7. Correlation between the concentration of cortisol in adrenal tissue and the rate at which it had been secreted into the adrenal vein immediately before the excision of the gland. Observations made on three pigs (●) and on five dogs (two adults: × ; three puppies: ○).

records observations made on the three puppies (see also Tables 2 and 5). The centre compartment in Fig. 8 represents the adrenal tissue, the two outside compartments the adrenal venous blood, and the arrows crossing the division lines indicate secretion into the adrenal vein. In the gland compartment the steroids are arranged in the sequence in which they are most likely to be synthesized. The 17-oxo steroids are confined to a separate space because their synthesis could also be accomplished along a pathway which by-passes pregnenolone. The amount of steroid is given by the area underneath or next to its name. In the gland compartment the areas represent concentrations, in the blood compartments secretion rates. The figures for pregnenolone, progesterone and 17αOH-progesterone are corrected for losses. The figures for the C-21 hydroxysteroids are underestimated by about 20 % and for other steroids by about 40 %.

The diagram shows that the quantities of steroids contained in the adrenal gland are small when compared with the secretion rates. The amounts of cortisol present in the adrenals of individual puppies were equivalent to the amounts secreted in 0·74, 0·71 and 0·83 min. This value ranged from 1·0 to 1·7 min for corticosterone and from 2 to 7 min for compounds A, S and E and the 17-oxo steroids.

The situation was somewhat different for the precursor steroids pregnenolone, progesterone and 17αOH-progesterone. These steroids were con-

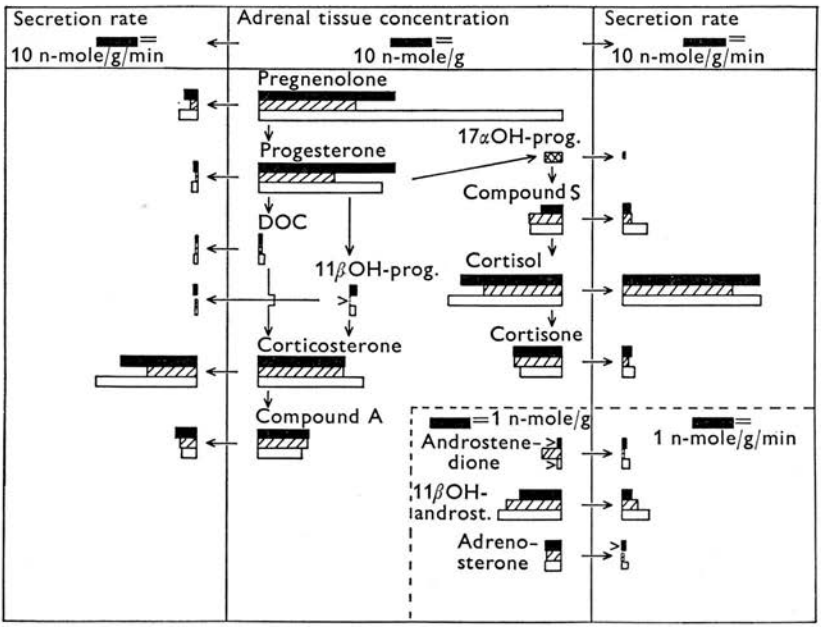


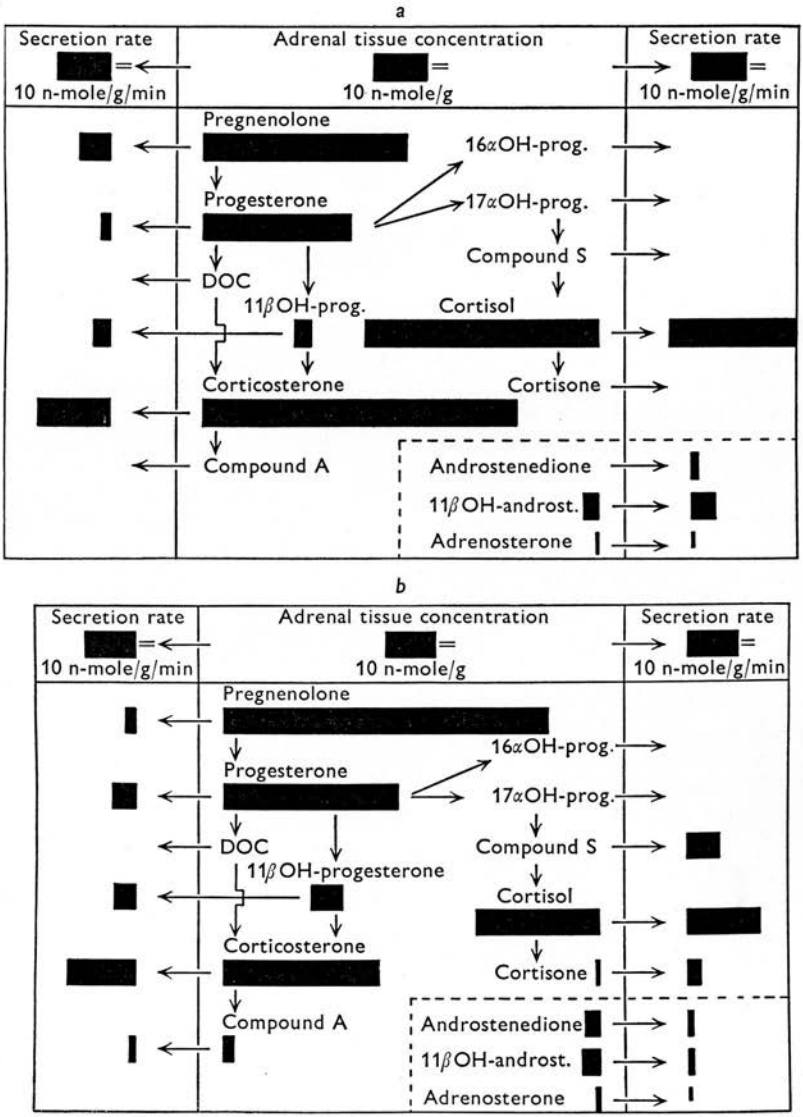
Fig. 8. Comparison of the concentrations of different pregnane and androstane derivatives in the adrenal gland (centre compartment) with the rates at which they were secreted during a period of 30 min preceding the excision of the gland (outside compartments). In the gland compartment the steroids of the pregnane series are arranged in the most likely sequence of formation. The arrows crossing the division lines between the gland and the blood compartment indicate secretion. The quantity of a steroid is represented by the area underneath or beside its name. Prog. = progesterone; androst. = androstenedione.

Puppies, 10-week-old litter, 3 kg, eviscerated and nephrectomized. ■ = puppy No. 1, ♂, chloralose anaesthesia, one adrenal blood sample collected. ▨ = puppy No. 2, ♀, pentobarbitone sodium anaesthesia, one adrenal blood sample collected. □ = puppy No. 3, ♀, pentobarbitone sodium anaesthesia; two adrenal blood samples collected; α-ethyltryptamine infused between samples 1 and 2, 60 min before excision of the gland. ⊠ = 17α-OH-progesterone, mean value of all three animals. Blood withdrawn replaced by donor blood. Estimates for pregnenolone and progesterone corrected for losses.

tained in the gland tissue in amounts which were equivalent to those secreted in 10–20 min. The progesterone concentrations in the adrenals were similar to those of cortisol or corticosterone. It was, however, secreted at a much slower rate. The adrenal concentrations of pregnenolone were larger than the progesterone concentrations in puppies Nos. 2 and 3, and the pregnenolone secretion rates were higher than the progesterone secretion rates in all three puppies. The amount of pregnenolone contained in the glands was equivalent to the sum of the pregnane derivatives secreted in 0·5–1 min. The adrenal concentration and secretion of

17α-OH-progesterone was much smaller than that of pregnenolone and progesterone.

Figure 9 illustrates observations made on three young pigs. For those steroids which were not analysed quantitatively the bar is omitted in the figures. The estimates for pregnenolone and progesterone are not corrected for losses. From pig No. 9 (Fig. 9*a*) and pig No. 14 (Fig. 9*b*) only one adrenal blood sample was collected for 5 and 10 min; the blood pressure



Figs. 9*a* and 9*b*. For legend see opposite page.

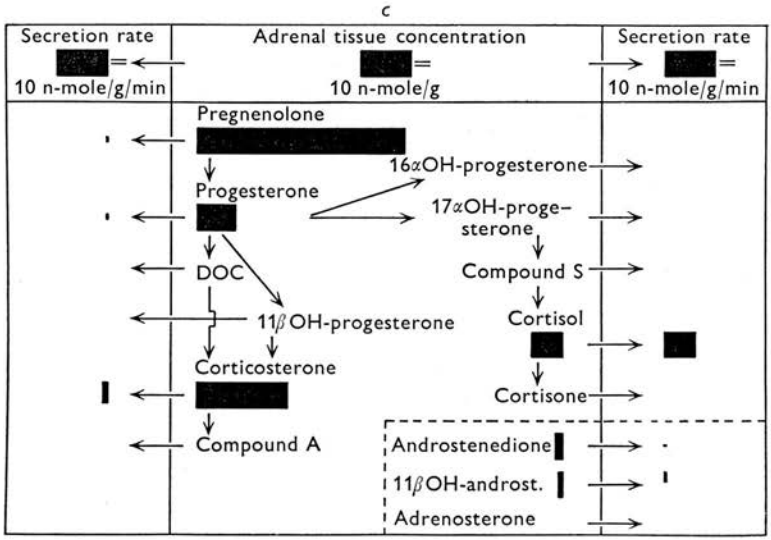


Fig. 9. Comparison of the concentrations of pregnane and androstane derivatives in the adrenal gland (centre compartment) with the rates at which they were secreted during the 10 (pig No. 9) or 5 (pigs Nos. 14 and 15) min period preceding excision of the adrenal glands (outside compartments). Diagram arranged as in Fig. 8. Only name of steroid given: found to be present but not measured. Estimates not corrected for losses. All pigs were anaesthetized with chloralose, eviscerated and nephrectomized. In pigs Nos. 9 and 14 the blood withdrawn was replaced by pig's blood.

(*a*) Pig No. 9, ♀, 12 weeks, 21 kg. One adrenal blood sample collected. Duration of experiment: 210 min.

(*b*) Pig No. 14, ♀, 11 weeks, 21 kg, premedicated with phencyclidine HCl. B.P. 85 mm Hg. Duration of experiment: 210 min.

(*c*) Pig No. 15, litter mate of No. 14, ♀, 12 weeks, 20 kg, premedicated with phencyclidine HCl. Received α-ethyltryptamine 60 min, and ACTH 25 min before excision of the glands. Both drugs were without effect on steroid secretion (see Fig. 6). Six adrenal blood samples collected, blood withdrawn replaced first (samples 1 and 2) by pig's blood, then by dextran. Secretion rates during the last 5 min blood collection period illustrated. Blood pressure during this period 45 mm Hg, haematocrit 4·1 %. Duration of experiment: 325 min.

was above 80 mm Hg and the haematocrit normal. In both animals the duration of the experiment from the induction of the anaesthesia to the removal of the adrenal glands was 210 min. In these two stressed, but otherwise 'normal' pigs, the ratios of adrenal concentrations to secretion rates were similar to those seen in the puppies under similar conditions for most steroids. The adrenal concentrations of 11βOH-progesterone were higher in the pigs and this steroid was also secreted at a faster rate by the pig.

Six adrenal blood samples were collected from pig No. 15 (Fig. 9*c*) and the haematocrit was only 4·1 % at the end of the last collection period. The

secretion of all steroids measured was severely decreased during the sixth collection period (see Fig. 6). As can be seen in Fig. 9*c*, the adrenal concentration of most steroids was also low. However, only for cortisol was it decreased to a similar degree as its secretion rate during the last collection period. The amount of corticosterone present in the gland was in fact quite large and equivalent to the amount secreted in about 20 min. The secretion of pregnenolone had nearly ceased. In spite of that, the amount of pregnenolone contained in the gland was within the normal range.

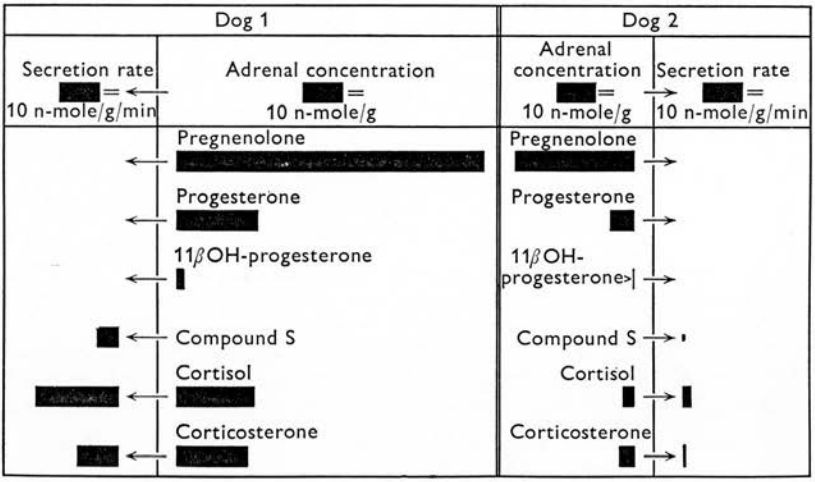


Fig. 10. Observations on dogs Nos. 1 and 2. Concentration of steroids in the adrenal glands (centre compartments) and secretion rates of glucocorticoids during a period of 3 min preceding excision of the glands (outside compartments). Blood collected from the left adrenal gland only. Both dogs received α-ethyltryptamine 60 min before the end of the experiment. Dog No. 1: ♀, 11 kg, chloralose anaesthesia; dog No. 2: ♀, 11 kg, pentobarbitone sodium anaesthesia.

Figure 10 illustrates observations made on two dogs. These experiments were designed to study the effect of different anaesthetics on the action of α-ethyltryptamine and only the secretion rates of compound S, cortisol and corticosterone were measured (see Table 3). After the infusion of the drug these rates were found to be decreased in dog No. 2, which was anaesthetized with pentobarbitone sodium, but not in dog No. 1, which was anaesthetized with chloralose. It can be seen that in the dog with inhibited secretion, the adrenal concentrations of all steroids measured were also lower. However, as in pig No. 15, the steroid concentrations in the gland were not decreased to the same degree as their secretion rates. In dog No. 1 the ratio between cortisol content and its secretion per min was 0·9, in dog No. 2 it was 1·5. For corticosterone the ratio was 1·7 in dog No. 1 and 5 in dog No. 2. The adrenal concentration of pregnenolone in dog No. 2,

although lower than in dog No. 1, was still within the normal range (compare Table 5); the progesterone concentration was lower than in the 'normal' stressed dog.

DISCUSSION

The steroid compounds pregnenolone, progesterone, 11βOH-progesterone and 11βOH-androstenedione were consistently found to be present during stress in the adrenal venous blood of the young pig and the puppy in concentrations which were considerably higher than their concentrations in the arterial blood of the same animals. In addition, androstenedione and adrenosterone were secreted by the adrenals of most animals. In the dog the secretion of all these steroids was previously found to be decreased after hypophysectomy, but it did not cease completely. This indicates that they are also secreted by the non-stressed animal. Secretion could be stimulated by ACTH (Holzbauer & Newport, 1968*a*, *b*).

The present experiments were not designed to investigate those factors which might have a specific influence on the secretion of one or the other of these steroids. However, an interesting difference in the response of individual steroids to severe blood loss was seen in the experiment on pig No. 6. During the collection of the first three adrenal blood samples the blood volume decreased progressively and the blood pressure was as low as 30 mm Hg during the third collection period. This was accompanied by a fall in steroid secretion rates, with the exception of pregnenolone and 11βOH-androstenedione. On the contrary, the secretion of these two steroids increased and it fell again after infusing dextran. The fall in aldosterone secretion which occurred in this pig after blood loss was in agreement with previous observations on dogs, in which a similar reaction of aldosterone secretion was seen when the blood pressure was very low (Holzbauer & Vogt, 1966).

A comparison was made between the amounts of steroids secreted in relation to body weight. Puppies appeared to secrete more pregnenolone and cortisol per kilogram body weight than the adult dogs. However, the experiments on the adult dogs were carried out on animals which were not eviscerated or nephrectomized and the problem requires reinvestigation under conditions which are more comparable. The largest amounts (relative to body weight) of pregnenolone, progesterone and 11βOH-progesterone were secreted by the young pig. This species showed also the largest individual variations, probably due to the longer time (up to 2 hr) taken to perform the evisceration in the young pig. The same operation in the puppies could be completed in 10 min.

The physiological significance of the adrenal secretion of precursor steroids is still obscure. Masuda, Anderson, Henricks & Melampy (1967)

measured the secretion of progesterone in ovarian venous blood in stressed adult pigs during the oestrous cycle and during pregnancy. From the observations of these authors it can be calculated that the progesterone secretion from one ovary varied during the oestrous cycle between 0·6 and 30 μg/min and during pregnancy between 9 and 28 μg/min. In the present experiments on immature stressed pigs the secretion of progesterone from one adrenal gland varied between 0·2 and 3 μg/min. This indicates that under stress conditions the amount of progesterone secretion from the adrenal gland could contribute substantially to the total amount of progesterone available to the body.

An attempt to achieve 'chemical hypophysectomy' in the pig with α-ethyltryptamine failed. This failure may have been caused by the removal of viscera and kidneys as well as by the use of chloralose as an anaesthetic. Each of these procedures prevented the action of α-ethyltryptamine in the dog; so did ether anaesthesia, according to Tullner & Hertz (1964).

All steroids found in adrenal venous blood were also present in extracts from the adrenal glands of several species. The highest concentrations of pregnenolone and progesterone were found in the pig and the rat.

In a number of animals it was possible to study the relationship between the concentrations of different steroids in the adrenal gland and the rates at which they had been secreted prior to the removal of the glands. Any conclusions drawn from such studies are necessarily based on the assumption that the steroid content in the adrenal gland at the time of its removal from the body is representative of the period during which the adrenal venous blood had been collected. It is furthermore assumed that none of the steroids measured are contained in, or were secreted by, the adrenal in a conjugated form, as has been reported for dehydroepiandrosterone (Baulieu, Corpèchot, Dray, Emiliozzi, Lebeau, Mauvais-Jarvis & Robel, 1965).

The adrenal concentrations of most of the steroids measured were found to be high in those animals in which their secretion rates were high and vice versa. This is in agreement with earlier observations on rats in which high adrenal concentrations of corticosterone and progesterone were found when the animals had been stressed before the excision of the adrenals, and low concentrations in unstressed rats (Holzbauer, 1957; Holzbauer & Newport, 1967*b*). In the present experiments the best correlation between adrenal secretion rates and adrenal concentrations existed for cortisol, the major glucocorticoid secreted by the pig and the dog. Thus, the amount of cortisol present in the adrenal gland might be a good index for the rate at which it was secreted prior to the removal of the gland. In the stressed animal with normally functioning pituitary and adrenal glands, the amounts

of the major glucocorticoids present in the adrenal tissue were equivalent to the amounts which were secreted during a period of between 0·5 and 3 min. Similar observations were made for DOC, 11βOH-progesterone and the 17-oxo steroids.

A different situation existed for pregnenolone. In the pig the ratio between the concentration of pregnenolone in the adrenal gland and the rate at which it was secreted was variable; it was 6·6 in pig No. 9, and 28 in pig No. 14; in pig No. 15, the secretion of pregnenolone had nearly ceased, but the pregnenolone concentration in the adrenal tissue was still within the normal range. The turnover rate of pregnenolone was also decreased in pig No. 15 as indicated by the slow secretion rates of the two major pregnane derivatives, cortisol and corticosterone. This suggests that a storage mechanism for pregnenolone exists in the adrenal cortex. Secretion of pregnenolone could occur either on its transfer from the locus of its formation to its storage site or from its storage site to the enzymes by which it is metabolized. The selective rise in pregnenolone secretion observed in pigs in which the volume of blood withdrawn was not replaced by either donor blood or dextran (see Figs. 2 and 3) could be due to a break-down of the storage mechanism.

We would like to express our thanks to Professor W. Klyne (M.R.C. Steroid Reference Collection) for samples of crystalline steroids, to Dr R. P. Edkins (Organon Labs.) for a sample of ACTH, to Dr R. B. Heap for his help with the enzyme incubation and to Mr R. P. Hughes for his skilful technical assistance. We are indebted to Dr M. Vogt, F.R.S., for her interest in this work. The adrenal glands of the horse were obtained from Dr D. Chapman, Equine Research Station, Newmarket.

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Secretion of Pregn-4-ene-3,20-dione (Progesterone) *in vivo* by the Adrenal Gland of the Rat

Feder, Resko and Goy[1] suggested recently that the adrenal gland of the rat secretes progesterone. They observed that the arterial blood of female rats still contained significant amounts of progesterone 25 days after bilateral ovariectomy; 8 h after additional adrenalectomy, however, the steroid was no longer detectable. Because the rat is often used for experiments in reproductive physiology, it is important to assess the contribution of the adrenal gland to the total available progesterone in the female rat. We have therefore measured the secretion of progesterone from the adrenal gland by estimating the amount of the steroid secreted into the adrenal venous blood. Experimental conditions were maintained comparable with those used recently for measuring the secretion of progesterone by the ovary of the rat[2–4].

Virgin female Wistar rats, 6 weeks (110–130 g) or 9 weeks (170–230 g) old, were used. Venous blood was collected from the left adrenal gland under pentobarbitone sodium anaesthesia (50 mg/kg) for 30 min, using the method described by Vogt[5]. Results are included from rats with adrenal glands which had been demedullated for other reasons 19–44 days before the adrenal blood was collected. Blood pressure was recorded from a carotid artery and supported by infusing either rat blood or a 0·9 per cent solution of sodium chloride. Before adrenal vein cannulation the arterial pressure ranged from 113 to 165 mm Hg; when blood was being collected it was lower (65–100 mm Hg). There was no significant difference between rats with intact or demedullated adrenal glands. In 30 min 2–3 ml. of adrenal blood was collected. To correct for losses during the chemical procedures 4-^{14}C-progesterone (0·4 mμCi) was added to each blood sample. The whole blood was extracted with a mixture of ethyl-acetate and ether (2 : 1, v/v), purified and applied to 2 cm wide strips of Whatman No. 2 chromatography paper. A descending chromatogram was developed for 40 h at 27° C in the toluene-propylene glycol (TPG) system of Zaffaroni and Burton[6]. The overflow which contained the progesterone was rechromatographed on sodium hydroxide-washed Whatman No. 50 paper in the E_1-system of Eberlein and Bongiovanni[7]. Progesterone was identified and estimated by gas–liquid chromatography in eluates from the E_1-chromatogram as described before[8]. Corticosterone was estimated according to its reaction

Table 1. SECRETION OF PREGN-4-ENE-3,20-DIONE (PROGESTERONE) BY THE ADRENAL GLAND OF THE RAT

Rat No.	Left adr. gland (mg)	Steroid secretion rates: Progesterone (μg/left adr./h/ 100 g b.wt.)	Steroid secretion rates: Progesterone (μg/h/ 100 mg adr.)	Steroid secretion rates: Corticosterone (μg/h/ (100 mg adr.)	Phase of oestrous cycle
Adrenal glands intact					
C1	25·4	0·28	2·13	144	—
C2	25·2	0·17	1·11	117	—
C3	26·0	0·26	1·69	86	Pro-oestrus
C4	35·8	0·61	3·24	196	Oestrus
C6	31·3	0·16	1·02	76	Oestrus
C7	15·9	0·23	1·89	61	Pro-oestrus
C8	21·7	0·43	2·49	115	Oestrus
	Mean ± *S.E.*:	0·31 ± 0·06	1·94 ± 0·29	114 ± 17	
Adrenal glands demedullated					
D1	13·4	0·07	1·00	65	—
D2	25·4	0·72	4·88	293	—
D3	25·6	0·16	1·25	173	Oestrus
D4	34·2*	0·28	1·64	174	Met-oestrus
D5	25·6	0·44	3·59	149	Met-oestrus
D6	30·0	0·31	2·33	191	Vagina artretic
D7	6·8	0·12	2·06	44	—
D8	10·4	0·16	1·92	204	Met-oestrus
	Mean ± *S.E.*:	0·28 ± 0·08	2·33 ± 0·46	162 ± 28	

	Left ovary mean: (mg)	Ovarian progesterone secretion rates† Progesterone secretion rates (μg/left ovary/h/ 100 g b.wt.) (mean ± *S.E.*)	(μg/h/ 100 mg ovary) (mean ± *S.E.*)	(Range)	
n = 8	38·8	1·81 ± 0·59	9·35 ± 3·9	2·16 – 33·30	Oestrus, met-oestrus
n = 4	36·8	< 0·11	< 0·52		Early pro-oestrus

Virgin female Wistar rats, 6 weeks (C1–C6, D1–D6, 110–130 g b.wt.) or 9 weeks (C7, C8, D7, D8, 170–230 g b.wt.); venous blood was from the left adrena gland collected under pentobarbitone sodium anaesthesia between 1000 and 1500 for periods of 30 min; body temperature was 37–37·8° C; C; control rats; D: rats with demedullated adrenal glands. Days after demedullation: D1, D2: 37; D3, D4: 43; D5, D6: 44; D7, D8: 19; —: not recorded; adr.: adrenal gland; b.wt.: body weight.

* Right adrenal removed.

† Results obtained in previous experiments on rats of the same strain (mean b.wt. 180 g) in similar experimental conditions[3].

with tetrazolium blue on eluates of the corresponding regions of the TPG chromatogram.

The results are given in Table 1. Progesterone was detected in the adrenal venous blood of all rats studied, except rat D1. The concentrations of progesterone in the adrenal venous blood varied between 0·04 and 0·15 μg/ml. Progesterone could not be detected in 5 ml. of arterial blood (< 0·03 μg/ml.) by the method used. The rates of secretion of corticosterone were of the same order of magnitude as those found earlier[9] and about fifty times greater than the secretion of progesterone.

Mean values of rates of ovarian progesterone secretion, obtained by Fajer and Holzbauer[3], who collected ovarian venous blood from rats of the same strain in similar experimental conditions, are included in Table 1. Comparison of these ovarian secretion rates of progesterone with adrenal progesterone secretion shows that, in conditions of surgical stress, the adrenal gland contributes a large proportion of the total progesterone available to the female rat. These results agree with the observations and conclusions of Feder *et al*[1].

The pig is another animal in which the adrenal gland can contribute a considerable proportion of the progesterone available to the body[10]. The adrenal secretion of progesterone has been found to vary between 0·2 and 3 μg/min. In similar conditions of stress, the secretion of progesterone from the ovaries during the oestrous cycle was reported to vary from 0·6 to 30 μg/min[11].

Further experiments were carried out with rats subjected to adrenal demedullation, for the pattern of steroid secretion might be modified by this operation. The results show that the adrenal secretion of corticosterone and progesterone was of the same magnitude in the absence of the adrenal medulla.

We thank Dr M. Vogt for carrying out the adrenal demedullations and for her interest in this work.

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Pregnenolone, progesterone and 20-dihydroprogesterone in rat ovarian blood and ovaries during the oestrous cycle

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The venous effluent from the left ovaries of mature virgin rats (mean body weight 180 g) was collected under pentobarbitone anaesthesia (Fajer & Barraclough, 1967) and both ovaries were removed at the end of the blood collection. The blood and the ovaries of each individual rat were analysed for their content of 3β-hydroxypregn-5-en-20-one (pregnenolone), pregn-4-ene-3,20-dione (progesterone) and 20α-hydroxypregn-4-en-3-one (20-dihydroprogesterone). The steroids were assayed using gas–liquid chromatography after purification and separation in two paper chromatographic systems (Holzbauer & Newport, 1967). The results are shown in Table 1.

TABLE 1. Ovarian secretion and contents of 3β-hydroxypregn-5-en-20-one (pregnenolone), pregn-4-ene-3,20-dione (progesterone) and 20α-hydroxypregn-4-en-3-one (20-dihydroprogesterone) in rats during the oestrous cycle

Rat no.	Phase of cycle*	Weight of both ovaries (mg)	Ovarian secretion rates (μg/g ovary/min)			Ovarian contents (μg/g ovary)		
			Pregnenolone	Progesterone	20-Dihydroprogesterone	Pregnenolone	Progesterone	20-Dihydroprogesterone
1	PE_1	69	< 0·02	< 0·03	< 0·06	4·64	5·07	14·36
2	PE_1	61	< 0·04	< 0·06	0·85	6·23	6·23	20·33
3	PE_2	88	< 0·03	< 0·06	0·28	5·90	12·20	26·60
4	PE_2	67	< 0·04	< 0·20	0·61	5·07	8·94	14·90
5	E	63	1·33	0·58	3·86	22·54	1·27	9·84
6	E	85	0·61	0·58	3·36	13·90	6·37	12·90
7	E	72	0·07	0·36	8·60	3·75	3·20	53·10
8	E	80	0·31	0·40	5·56	9·63	35·00	25·38
9	ME	63	0·51	1·25	9·98	7·04	7·68	11·00
10	ME	56	0·54	5·55	9·94	2·76	5·05	11·37
11	DE	84	0·26	1·43	7·80	12·26	28·57	8·69
12	DE	70	0·17	2·55	11·83	7·29	28·57	8·70

* Phases of oestrous cycle assessed by the cytology of the vaginal smear. PE = pro-oestrus; nucleated epithelial cells only. PE_1: blood collected before 11 a.m., PE_2: blood collected after 3 p.m. E = oestrus; cornified cells only. ME = met-oestrus: a great number of leucocytes. DE = di-oestrus: very few cells.

The rates at which progesterone and 20-dihydroprogesterone were secreted could be correlated with the cytological picture of the vaginal

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smears and thus with the different phases of the oestrous cycle. During the periods preceding ovulation (rats 1–4) these steroids were hardly detectable in the ovarian blood. After ovulation (rats 5–12) ovarian secretion started and reached a maximum during met- and di-oestrus. Pregnenolone was also found in the ovarian blood during these secretory phases.

The mean concentration of C-21 steroids in the ovarian tissue did not show significant differences between the periods before and after ovulation although individual variations were greater after ovulation.

It appears that at the time of ovulation a hormone is supplied which stimulates the synthesis of C-21 steroids. During the non-secretory phase there would be lack of this hormone and thus cessation of steroid synthesis; in addition there is regression of luteal tissue. When secretion starts, ovarian C-21 steroid content did not consistently rise. In individual rats it covered the amounts secreted over a period of 6–11 min during oestrus, and of 1–5 min during met-oestrus and di-oestrus.

The ovary, like the adrenal gland (Holzbauer & Newport, 1967), does not contain any excess of pregnenolone, the immediate precursor of progesterone. In ovarian steroid synthesis an increase in the rate at which pregnenolone is synthesized is therefore also the prerequisite for an increase in the secretion and synthesis of C-21 steroids.

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THE EFFECT OF STRESS ON THE CONCENTRATION OF 3β-HYDROXYPREGN-5-EN-20-ONE (PREGNENOLONE) AND PREGN-4-ENE-3,20-DIONE (PROGESTERONE) IN THE ADRENAL GLAND OF THE RAT

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SUMMARY

1. Pregnenolone and progesterone were estimated in extracts from adrenal glands of rats by gas–liquid chromatography.

2. In unstressed rats their concentrations were found to be smaller than that of corticosterone.

3. Acute stress caused an increase in the concentrations of these steroids which paralleled that of corticosterone.

INTRODUCTION

In the rat, the amount of 11β,21-dihydroxypregn-4-ene-3,20-dione (corticosterone) present in the adrenal tissue is only sufficient to cover the amount secreted in 2–3 min. The relation between corticosterone content and secretion rate has been found to be constant under different experimental conditions. Thus, corticosterone concentrations in the adrenal were high after stressful stimuli or injections of adrenocorticotrophic hormone (ACTH), adrenaline and histamine and low in the unstressed animal (Holzbauer, 1957). The acute effect of ACTH on steroid secretion can therefore not be due to the activation of a mechanism which enables the release of preformed steroids from a storage site in the tissue, but must be due to an increase in the velocity at which corticosterone is formed.

The experimental evidence available at present (Dorfman & Ungar, 1965) supports the view that ACTH exerts its major actions on the processes which are involved in the transformation of cholesterol to 3β-hydroxypregn-5-en-20-one (pregnenolone) (Stone & Hechter, 1954) and that its main function is to provide sufficient amounts of pregnenolone for the synthesis of corticosteroids. It seemed therefore of interest to study the adrenal

concentration of pregnenolone and its immediate metabolite pregn-4-ene-3,20-dione (progesterone), in resting rats and in rats in which endogenous ACTH release was stimulated by stress, such as exsanguination under ether anaesthesia or exposure to a cold environment. This type of investigation has become possible by the development of gas chromatographic methods which allow the quantitative estimation of nanogram amounts of these steroids.

METHODS

The experiments were carried out on adult Wistar rats weighing 150–200 g. They were kept in an isolated room and accustomed to handling during the 7 days preceding the experiment. The effect of exsanguination was studied on thirty-three female rats which were divided into four groups of six unstressed control animals and three groups of three stressed rats. On the day of the experiment the unstressed controls were killed by rapid decapitation. The stressed rats were anaesthetized with ether, a polythene cannula was inserted into a carotid artery and the rats were bled until respiration ceased. The adrenal glands of all rats were removed immediately after death and the glands of each group of rats pooled and homogenized. In the experiment in which the effect of cold exposure was studied fifteen male rats of a different colony were used which were divided into one group of six unstressed control animals and three groups of three stressed rats. The first group of stressed rats was kept at $-10°$ C for 5 min, the second group for 15 min and the third group for 90 min. They were decapitated immediately afterwards, and the adrenals of each group were pooled and homogenized. Exposure to $-10°$ C is well tolerated by rats for periods of more than 24 hr (J. LeBlanc, personal communication). None of the animals used in the present experiments was hypothermic; those kept at $-10°$ C for 90 min showed piloerection. The control rats were decapitated on the same morning and their adrenals were also pooled.

Extraction of the adrenal tissue and purification of the extracts were carried out as described previously (Holzbauer, 1957). To separate individual steroids, the extracts were first applied to 2 cm lanes of Whatman No. 2 paper which had been washed with the mixture used for elution (ethylacetate:methanol, 2:1), and a chromatogram was developed for 4 hr in the $B_5(a)$ system (Bush & Sandberg, 1953). In this system, corticosterone, the major C_{21}-steroid produced by the rat adrenal, has an R_F value of 0·75 and 18,21-dihydroxypregn-4-ene-3,20-dione (18OH-DOC) which is also produced by the rat in appreciable amounts (Birmingham & Ward, 1961; Péron, 1961) an R_F of 0·70. The regions containing these two compounds were eluted together and the steroids estimated by their reaction with blue tetrazolium (Vogt, 1955). The results were expressed in nanomoles of corticosterone, as 18OH-DOC does not reduce blue tetrazolium readily.

Pregnenolone and progesterone were contained in the region between corticosterone and the solvent front. For their further purification the eluates of these regions were rechromatographed with the E_1 system (Eberlein & Bongiovanni, 1955). Whatman No. 50 paper was used which was prepared as described by Holzbauer & Newport (1967). In the E_1 system, the R_F value of pregnenolone was 0·35 and that of progesterone 0·50. They were well separated from 11β-hydroxyandrost-4-ene-3,17-dione (11βOH-androstenedione, R_F 0·10) which had been provisionally identified in rat adrenal vein blood (Bush, 1953).

For further analysis the eluates of the different regions of this chromatogram were subjected to gas–liquid chromatography on a Model 402 F & M gas chromatograph. A 120 cm U-shaped glass column (4 mm i.d.) packed with 3·8 % SE–30 on silanized Diatoport S (60–80 mesh) at 230° C, a flame ionization detector (260° C) and argon as carrier gas (flow rate: 50 ml./min) were used. The detector responses were recorded on a Honeywell recorder. The retention times of the steroids were expressed in relation to that of cholestane which averaged 20 min and were referred to as relative retention times (RRT). All samples were applied to the column in ethanolic solutions in volumes of 1–2 μl.

The quantitative estimation of pregnenolone and progesterone was carried out by gas–liquid chromatography according to the method described by Heap, Holzbauer & Newport (1966) using 11βOH-progesterone as reference steroid. This method is based on the straight line relation which exists between the concentration ratio of steroids in a solution and the ratio of the peak heights of the recorded responses, observed when this solution is injected into the gas chromatograph. The dried eluates of the pregnenolone and progesterone regions were first dissolved in 40 μl. of pure ethanol and 1 μl. injected into the gas chromatograph to establish whether pregnenolone (RRT 0·62) or progesterone (RRT 0·81) were present and whether any compound produced a peak which would interfere with that of the chosen

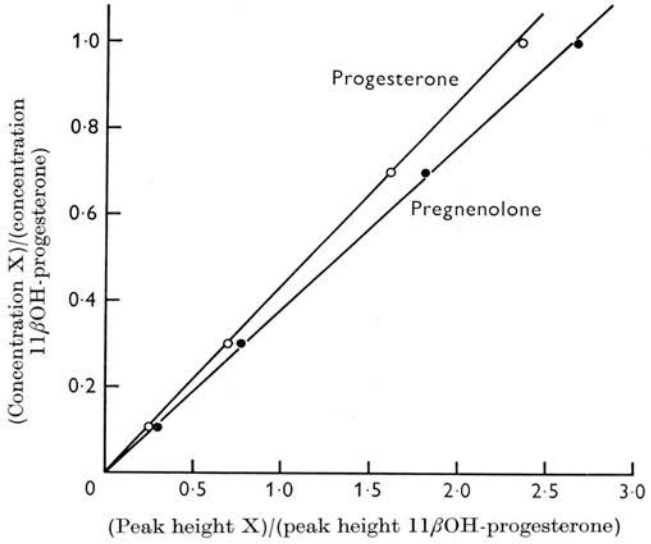

Fig. 1. Calibration curve for the gas chromatographic estimation of pregnenolone and progesterone using 11βOH-progesterone as reference standard (120 cm 3·8 % SE–30 column; X = pregnenolone or progesterone). (Coefficients of variance between 4·5 and 7·5 %.)

reference steroid 11βOH-progesterone (RRT 1·42). The ethanol was then evaporated and the residue taken up in an exact volume (10–40 μl., depending on the amount of steroid present) of a solution containing 100 μg 11βOH-progesterone/ml. ethanol. Volumes of 1–2 μl. were applied to the column of the gas chromatograph. The height of the recorded pregnenolone or progesterone peak was measured and divided by that of the reference steroid giving the peak height ratio. On the same day calibration lines were prepared with four mixtures of authentic progesterone, pregnenolone and 11βOH-progesterone in ethanol in which the concentration ratios of progesterone or pregnenolone to 11βOH-progesterone were 0·1, 0·3, 0·7 and 1·0 (Fig. 1). The amount of pregnenolone or progesterone in an eluate was calculated from the peak height ratios by deriving the corresponding concentration ratios from the calibration curve (Fig. 1) and multiplying this figure by the amount of 11βOH-progesterone added to the dry eluate. In this way 50 ng of steroid per sample could still be measured with fair accuracy. When it was established that extracts of about 300 mg of rat adrenal tissue did not contain detectable amounts of androst-4-ene-3,11,17-trione (adrenosterone) 5 μg of this steroid were added to each homogenate to assess the losses during the purification and chromatographic procedures. The amounts finally recovered averaged 60 %. This figure was confirmed in a later set of experiments in which [4-^{14}C]-

pregnenolone and [4-^{14}C]-progesterone were added to gland extracts. The radioactivity present in an aliquot of the solution used for the assay was then measured. Although the losses of adrenosterone seemed to be similar to those of pregnenolone and progesterone, the results obtained in the present experiments were not corrected for losses. The amounts of corticosterone recovered averaged 80 %.

RESULTS

Qualitative observations

Pregnenolone. The regions with R_F values corresponding to those of pregnenolone and progesterone were eluted from the E_1-chromatograms and the dried eluates dissolved in 40 μl. of ethanol. Figure 2 shows an example of the gas chromatograph tracings which were obtained when the

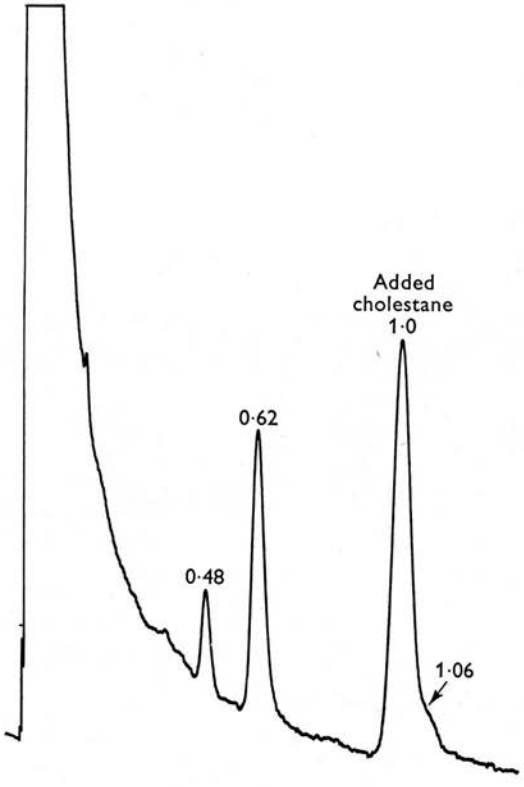

Fig. 2. Gas chromatograph tracing. Extract from 140 mg adrenal tissue from stressed rats, chromatographed in B_5 (*a*) and E_1. Region with R_F value of pregnenolone eluted, eluate dried, dissolved in 40 μl. ethanol, 1 μl. applied to the column (range 1, attenuation 8).

The numbers are retention times relative to that of cholestane (RRT). Authentic pregnenolone had a RRT of 0·62. The shoulder at the bottom of the cholestane peak is due to the 'paper blank', and had a RRT of 1·06. (RRT 0·48: possibly androst-4-ene-3,17-dione).

eluates of the pregnenolone regions were injected. All eluates produced a peak at RRT 0·62, corresponding to that of authentic pregnenolone and some of them also a peak at RRT 0·48 corresponding to androst-4-ene-3,17-dione. In addition, the gas chromatograph tracings showed a peak at RRT 1·06 which was always obtained when eluates from this type of

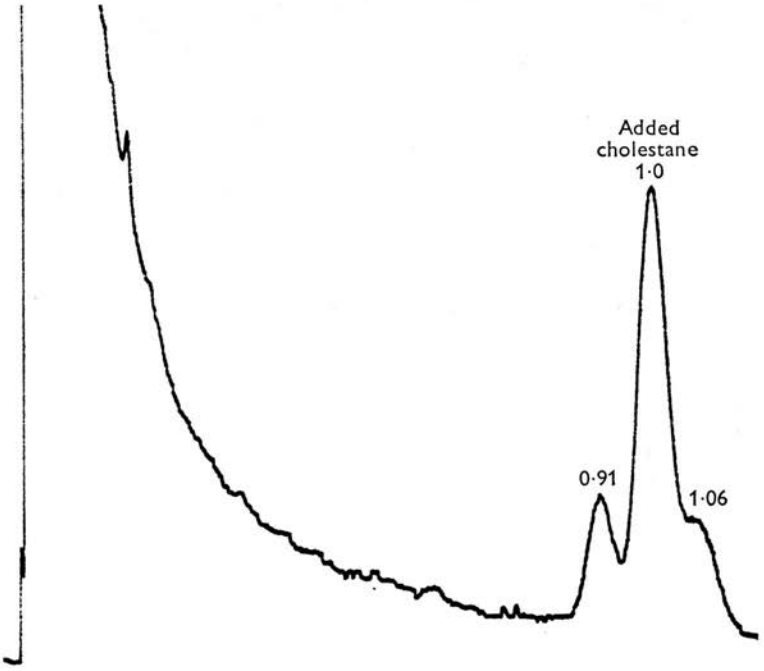

Fig. 3. Gas chromatograph tracing. Eluate of pregnenolone region of first E_1 chromatogram (see Fig. 2) acetylated and rechromatographed in E_1. Region with R_F value of pregnenolone acetate eluted, eluate dried, dissolved in 40 μl. ethanol, 1 μl. applied to the column (range 1, attenuation 8).

The numbers are retention times relative to that of cholestane (RRT). Authentic pregnenolone acetate had a RRT of 0·91. RRT 1·06:'paper blank'.

chromatography paper were applied ('paper blank'). Cholestane was injected simultaneously to enable accurate assessment of the RRTs. After the amount of pregnenolone contained in the individual samples had been estimated, the eluates were acetylated and rechromatographed for 5 hr in the E_1-system. The eluate of the region with the same R_F value as pregnenolone acetate (0·9) showed on gas chromatography a peak at RRT 0·91 (Fig. 3), the RRT of authentic pregnenolone acetate. The eluate of the region corresponding to pregnenolone showed only the small paper blank peak of RRT 1·06.

Progesterone. Figure 4 shows an example of the gas chromatography records which were obtained when the eluates of the progesterone regions

were applied. All eluates produced a peak at RRT 0·81, like that of authentic progesterone. In addition there were usually two minor unidentified peaks present. After the quantitative estimation of progesterone several samples were combined and incubated with the enzyme 20β-hydroxysteroid dehydrogenase (Boehringer, Mannheim), under conditions whereby the keto group in position 20 is reduced to a β-hydroxyl group (Henning

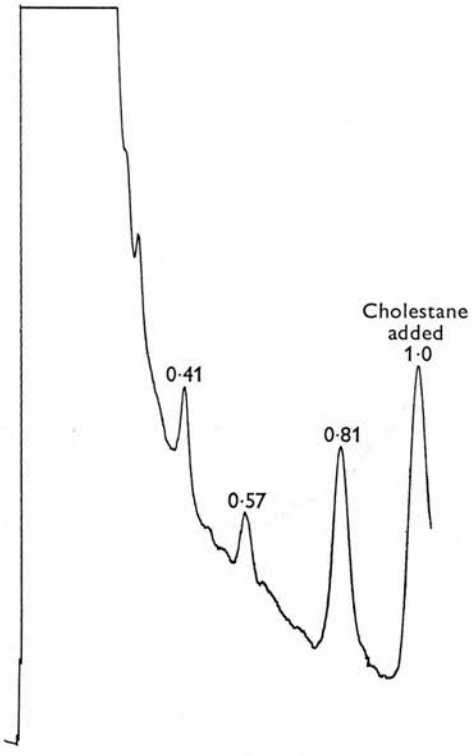

Fig. 4. Gas chromatograph tracing. Extract from 120 mg adrenal tissue from stressed rats, chromatographed in B_5 (*a*) and E_1. Region with R_F value of progesterone eluted, eluate dried, dissolved in 40 μl. ethanol, 1 μl. applied to the column (range 1, attenuation 8).

The numbers are retention times relative to that of cholestane (RRT). Authentic progesterone had a RRT of 0·81. (RRT 0·41 and 0·57: unidentified.)

& Zander, 1962; Heap, 1964). The RRT of this steroid is only slightly greater than that of progesterone. However, the hydroxysteroid can be acetylated to 20β-hydroxypregn-4-en-3-one acetate which has a RRT of 1·27. Thus, after enzymic conversion, the sample was acetylated and rechromatographed for 5 hr in the E_1-system. When the eluate of the region with an R_F value similar to that of 20β-hydroxypregn-4-en-3-one acetate

(0·95) was subjected to gas chromatography a peak at RRT 1·27 was obtained. No progesterone was left in the progesterone region of this chromatogram.

21-*Hydroxypregn*-4-*ene*-3,20-*dione* (DOC). Eluates of the region with an R_F value similar to that of DOC produced on the gas chromatograph tracing a peak at RRT 1·52, like authentic DOC. On acetylation the peak

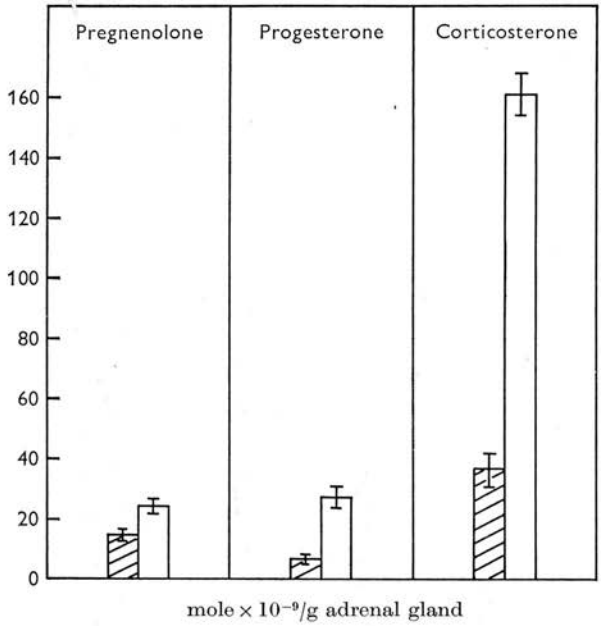

Fig. 5. Concentration (mole × 10^{-9}/g gland ± S.E.) of pregnenolone, progesterone and corticosterone in the adrenal glands of unstressed and stressed female rats. ▨: unstressed rats: four groups of six rats each. □: stressed rats (exsanguinated under ether anaesthesia): three groups of three rats each.

Differences between unstressed and stressed rats statistically significant. $P < 0.05$ for pregnenolone; $P < 0.01$ for progesterone and corticosterone.

moved to RRT 2·22, corresponding to that of DOC-acetate. After rechromatography in the E_1-system for 6 hr a U.V. absorbing spot was seen with the same R_F value as DOC-acetate and its eluate showed again a peak at RRT 2·22 on the gas chromatograph tracing.

Cholesterol. The eluates of the progesterone regions of all samples showed an additional large peak at RRT 2·0, corresponding to that of cholesterol. As happens with cholesterol, the RRT changed after acetylation to 3·0.

Quantitative observations

Exsanguination under ether anaesthesia. The results obtained on unstressed control rats and rats killed by bleeding from a carotid artery

under ether anaesthesia are summarized in Fig. 5. In the unstressed rats the amount of pregnenolone contained in the adrenal glands amounted to less than one half, that of progesterone to less than one quarter of the amount of corticosterone calculated on a molar basis. Ether anaesthesia and bleeding not only caused the corticosterone concentration but also that of progesterone, to increase more than threefold. The pregnenolone content rose by 65 %.

Cold stress. In Fig. 6 the results of experiments are summarized in which the stressful stimulus was graded by varying the length of time for which

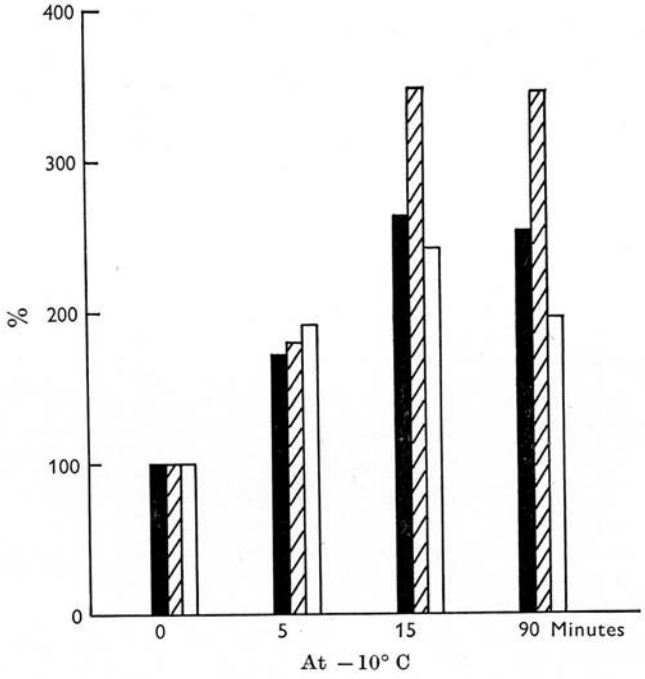

Fig. 6. Increase in the concentration of pregnenolone ■, progesterone ▨ and corticosterone □ in the adrenal glands of male rats after exposure to −10° C for 5, 15 or 90 min. The results are expressed as percentage of the steroid concentration in unstressed control rats. Each column represents the estimate for a pooled sample of the adrenal glands of six unstressed rats or three stressed rats.

rats were exposed to −10° C. The results are expressed as a percentage of the steroid concentration in the adrenals of the unstressed control rats. In this rat colony the ratios of the adrenal concentrations of corticosterone: progesterone and pregnenolone were even higher mainly due to a larger content of corticosterone. In the adrenal glands of the unstressed controls the corticosterone concentration was 180×10^{-9} mole/g tissue, that of pregnenolone $4{\cdot}5 \times 10^{-9}$ mole/g and that of progesterone $8{\cdot}1 \times 10^{-9}$ mole/g.

Substantial differences in the corticosterone concentrations in adrenals of rats belonging to different colonies are well known. Like exsanguination, cold stress also caused a rise in the adrenal concentration of the three steroids. After 5 min exposure the concentration of all three steroids was doubled. After 15 min the concentrations were further increased. When the exposure to cold was extended to 90 min no further rises in the steroid concentrations were observed.

The observations on DOC were only semiquantitative. The concentrations were certainly lower than those of corticosterone.

DISCUSSION

In unstressed rats the amounts of pregnenolone and progesterone contained in the adrenal glands were found to be smaller than those of corticosterone, the major secretion product of the rat adrenal cortex. These observations indicate that the acceleration of the synthesis of C_{21}-precursor steroids is a prerequisite of any increase in the secretion of corticosterone. They are compatible with the view that the immediate action of ACTH takes place on a system (or systems) which accelerate the formation of pregnenolone. This is reflected by the rise in the adrenal concentration of pregnenolone shortly after the onset of stress. Exposure to cold environment for 5 or 15 min resulted in concomitant increases in the concentrations of pregnenolone, progesterone and corticosterone. Extending the stress to 90 min did not lead to a further accumulation of any of the three steroids.

We would like to express our thanks to Professor W. Klyne (M.R.C. Steroid Reference Collection) and to Dr K. E. Meyer (Ciba Ltd., Basle, Switzerland) for samples of crystalline steroids, to Dr R. B. Heap for his help with the enzyme incubation and to Mr R. Hughes for his technical assistance. We are indebted to Dr M. Vogt, F.R.S., for her interest in this work. The experiments on the rats exposed to cold were done in collaboration with Dr J. LeBlanc in the course of an investigation into the effect of cold on adrenergic mechanisms.

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14

THE ACTION OF CHLORPROMAZINE ON DIENCEPHALIC SYMPATHETIC ACTIVITY AND ON THE RELEASE OF ADRENOCORTICOTROPHIC HORMONE

BY

MARGARETHE HOLZBAUER and MARTHE VOGT

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THE ACTION OF CHLORPROMAZINE ON DIENCEPHALIC SYMPATHETIC ACTIVITY AND ON THE RELEASE OF ADRENOCORTICOTROPHIC HORMONE

BY

MARGARETHE HOLZBAUER AND MARTHE VOGT

From the Department of Pharmacology, University of Edinburgh

(RECEIVED AUGUST 28, 1954)

Chlorpromazine exerts a peculiar type of sedative action in man and in animals, potentiates and prolongs the action of analgesics and hypnotics, antagonizes excitation by alcohol and nikethamide, and protects animals from shock (for reference see Courvoisier, Fournel, Ducrot, Kolsky and Koetschet, 1953). To explain some of these effects, it has been suggested that the drug has an inhibitory action on sympathetic diencephalic centres (Laborit and Huguenard, 1951 ; Delay and Deniker, 1953) and that it prevents the release of adrenocorticotrophic hormone (ACTH) in conditions of stress (Aron, Chambon, and Voisin, 1953), an action which might also be interpreted as an inhibition of hypothalamic activity. The object of this paper is to investigate these possibilities.

METHODS

Hypothalamic Activity in Cats.—Six cats were subjected to left adrenal denervation, under ether anaesthesia, with aseptic precautions ; on the left side, both splanchnic nerves were severed and the first three lumbar sympathetic ganglia extirpated. In two of the cats, the left superior cervical ganglion was removed a fortnight later. Between two and three weeks after the first operation, the drugs under investigation were injected subcutaneously ; between 4 and 5 hr. after the injection of the first drug, the cats were rapidly anaesthetized with chloroform, bled to death, and their hypothalamus and adrenal glands removed for examination.

Morphine HCl and nalorphine HBr were injected as 2% solutions in 0.9% NaCl ; chlorpromazine was given as 0.15% solution, since higher concentrations have an irritant action.

Estimation of hypothalamic noradrenaline and of the amines of the adrenal medulla was carried out by biological assay on extracts subjected to paper chromatography ; the separated amines were eluted from the paper and the eluates assayed on the rat's blood pressure (urethane anaesthesia ; pretreatment with 2 mg./kg. atropine sulphate and 16 mg./kg. hexamethonium bromide intravenously). In some experiments the results of the adrenaline estimations were checked on the rat's uterus. All details of the methods have been reported earlier (Vogt, 1954).

Blood Pressure and Pupil Size of Dogs.—The action of chlorpromazine as an antagonist of adrenaline was followed on a dog anaesthetized with chloralose. The femoral blood pressure was recorded and observations were made of the effects on pupil size produced by the injection of drugs and by faradic stimulation of the preganglionic fibres of the severed cervical sympathetic trunk.

Experiments on the Adrenal Cortex of Rats.—The release of ACTH in conditions of stress was followed in rats by adrenal ascorbic acid estimations according to the method of Roe and Kuether (1943). One set of experiments was carried out in anaesthesia (1.75 g. urethane/kg. subcutaneously), and "operative stress" used to stimulate the release of ACTH. When the rat was anaesthetized an abdominal incision was made, the intestine exposed, and the mesentery handled for a period of 2 min. The wound was sewn up, the rat replaced in a warm box and killed, while still deeply anaesthetized, by severing the neck 1 hr. later ; the adrenals were then removed for ascorbic acid estimations. The effects obtained in normal rats were compared with those seen in rats injected subcutaneously with chlorpromazine 30 min. or 3 hr. before the anaesthetic.

In a second group of experiments, the fall in adrenal ascorbic acid following a subcutaneous injection of adrenaline was compared under normal conditions and 3 hr. after the subcutaneous injection of chlorpromazine. All rats used in this experiment had been accustomed to handling by being given subcutaneous injections of 0.9% NaCl for a preliminary period of one week.

RESULTS

Hypothalamic Activity in Cats.—For testing central sympathetic activity use was made of the fact that morphine, given to cats, causes stimulation of the hypothalamic sympathetic centres. This can readily be measured by denervating one adrenal and estimating the difference in amine con-

tent between the innervated and denervated (resting) gland elicited by an injection of morphine. A second index of sympathetic hypothalamic stimulation is the fall in the noradrenaline content of the tissue observed when the stimulus has been acting over a period of several hours (Vogt, 1954). If chlorpromazine was able to antagonize a central sympathetic stimulation, the effects of morphine in depleting the stores of amines in the innervated adrenal and of noradrenaline in the hypothalamus might be inhibited.

Table I illustrates the results. The first cat was used as a control and injected with chlorpromazine only. As might be expected, there was no secretion of adrenaline from the innervated adrenal, and the hypothalamic noradrenaline was not significantly different from normal. Cats 2, 3, and 4 were given 40 mg./kg. of morphine 45 min. after a first dose of 15 mg./kg. chlorpromazine; a second dose of chlorpromazine (10 mg./kg.) was injected about 2 hr. after the morphine. Hypothalamic noradrenaline was low, and there was secretion of amines from the innervated medulla; the figures are not different from those of the last row, which represent previous observations on cats given the same dose of morphine but no chlorpromazine.

Thus chlorpromazine, given in a high dose, had no antagonistic effect on the diencephalic stimulation of the sympathetic centres produced by morphine. In another experiment, this result was compared with the effect of a proved morphine antagonist, nalorphine, on the hypothalamic stimulation produced by the same dose of morphine. Cats 5 and 6 (Table I) were given an injection of morphine 12 min. after the subcutaneous administration of nalorphine. The fall in hypothalamic noradrenaline was completely prevented in both cats; a small stimulation of the innervated adrenal medulla was seen in cat 5, which had the smaller dose of nalorphine, whereas this effect was abolished in cat 6, which was given the larger dose. Suppression of the clinical signs too was more complete in cat 6, which showed no abnormality until nearly 5 hr. after the nalorphine: when it was picked up in order to be anaesthetized with chloroform, there was sudden excitation and tremor, indicating that the effect of the nalorphine was beginning to subside. Cat 5, in contrast, showed salivation and some muscular twitching throughout the experiment, though the animal was much quieter than would have been expected as a result of morphine alone.

The clinical picture after chlorpromazine followed by morphine was varied: cat 2 showed no muscular twitches and no motor excitement; instead, it was sitting still or lying down with its head drooping to one side. In cats 3 and 4 muscular twitching and tremor were not suppressed by chlorpromazine as they had been in cat 2. One short fit of convulsions was seen in cat 4.

One of the cats vomited, a fact which is of interest in view of the excellent anti-emetic action of chlorpromazine in the dog given apomorphine (Courvoisier *et al.*, 1953).

A striking sign, in view of the adrenolytic action of chlorpromazine, was, however, a dilatation of the pupils, whereas the nictitating membranes were relaxed. This was first seen in cat 2 and was obviously caused by the morphine (which is a mydriatic in cats), since the chlorpromazine control (cat 1) had narrow pupils and relaxed nictitating membranes.

In order to determine whether the mydriasis was a sign of circulating adrenaline, or was due to some other cause, the next two experiments (Nos. 3 and 4) were carried out on cats in which the left superior cervical ganglion had been re-

TABLE I

HYPOTHALAMIC NORADRENALINE, AND SECRETION FROM INNERVATED ADRENAL MEDULLA, AFTER SUBCUTANEOUS MORPHINE ALONE AND IN COMBINATION WITH CHLORPROMAZINE OR NALORPHINE

Cat No.	Chlorpromazine* (mg./kg.)	Nalorphine HBr (mg./kg.)	Morphine HCl (mg./kg.)	Hypothalamic Noradrenaline			Amines‡ in Innervated Adrenal Medulla (% of Denervated Medulla)	
				μg./g. Fresh Tissue	Mean (μg./g.)	% of Normal†	Individual Expts.	Mean
1	25	0	0	1·3		94	100	
2	25	0	40	0·8			75	
3	25	0	40	1·2	0·98	68	44	59
4	25	0	40	0·8			59	
5	0	25	30	1·4		101	78	
6	0	37·5	40	1·4		101	100	
Mean of 5	0	0	40		0·92	67	40, 57§	

* Given in two doses, interval 2½ hr. † The normal figure (a mean of 29 cats) is 1.38 μg./g. fresh tissue (Vogt, 1954). ‡ Adrenaline and noradrenaline in the adrenal medulla were estimated separately and added together. § 2 cats only.

The cats had been subjected to denervation of the left adrenal 2–3 weeks previously. Cat 1 was killed 5 hr. after the first injection of chlorpromazine; the other cats were killed 4½ hr. after the injection of morphine.

moved one week previously. The nictitating membranes of both cats remained relaxed throughout the experiment, but dilatation of both pupils—and, more strikingly, of the previously narrower, denervated pupil—occurred, particularly towards the end of the experiment. The obvious interpretation was that the pupils responded to circulating medullary amines and that the adrenolytic effect of chlorpromazine was not very powerful.

Antiadrenaline Effect.—The unexpected mydriasis in response to circulating adrenaline (and noradrenaline) in cats given chlorpromazine prompted a reassessment of the adrenolytic effect of chlorpromazine. Since the substance is credited with a ganglion-blocking action, its effect on transmission in a sympathetic ganglion was also tested. In the cats, response to circulatory medullary amines had been noticed as early as 80 and 95 min. after chlorpromazine in a dose of 15 mg./kg. An acute experiment was therefore carried out on a dog anaesthetized with chloralose, and the responses of blood presssure and pupil to injected adrenaline and noradrenaline, and to faradic stimulation of the preganglionic sympathetic fibres in the neck, were observed.

Between 5 and 15 min. after a first intravenous dose of chlorpromazine (1.5 mg./kg.), the effect of adrenaline (4 μg./kg.) on the blood pressure was reversed and that of the same dose of noradrenaline reduced; the mydriatic effects on the pupil had ceased. Preganglionic stimulation of the sympathetic chain, however, was as effective on the pupil as before. Half an hour after the administration of chlorpromazine, the effect of adrenaline began to recover, and a second, higher dose of chlorpromazine (3.0 mg./kg.) was injected. Adrenaline reversal on the blood pressure again ensued, but the threshold to electrical stimulation of the sympathetic chain remained unaltered. Two hr. 20 min. later, pressor and mydriatic effects of adrenaline were back to normal. The effect of sympathetic stimulation, hitherto unchanged, was then abolished by an injection of hexamethonium bromide (2.7 mg./kg.).

It follows from this experiment that the adrenolytic effect of chlorpromazine need not be as prolonged as is often supposed. As with other adrenaline antagonists, the effects of noradrenaline are more resistant to the drug than those of equal doses of adrenaline, and the effects of sympathetic stimulation are more resistant still. In addition, with the dosage (total of 4.5 mg./kg.) used, there was no sign of inhibition of transmission at the sympathetic ganglionic synapse. The fairly short duration of the adrenaline antagonism explains the observation on cats premedicated with chlorpromazine, in which pupillary dilatation in the sympathetically denervated eye occurred after a dose of morphine causing secretion of the adrenal medulla.

Release of ACTH in Stress.—The first part of this work dealt with possible effects of chlorpromazine on centrally initiated secretion by the adrenal medulla. The second part deals with the question whether the drug influences the stimulation of the adrenal cortex elicited by noxious or other stimuli capable of releasing ACTH.

In a first series of experiments, "operative stress" was employed in view of the fact that chlorpromazine protects animals from traumatic shock. The "stress" consisted in anaesthetizing the rats by subcutaneous injection of urethane, carrying out a laparotomy and handling the intestine. The results are shown in Table II.

In Groups 1–3 chlorpromazine was given in a dose of 10 mg./kg. and the operation was carried out 3 hr. later. The rats were killed and the adrenals removed at 4½ hr. The mean ascorbic acid content of the adrenals of Group 1, given chlorpromazine but not operated on, was 321 mg./100 g., and that of the rats operated on without pretreatment (Group 2) was 212 mg./100 g.

TABLE II

THE EFFECT OF CHLORPROMAZINE ON THE ASCORBIC ACID CONTENT (MG./100 G. FRESH ADRENAL) OF THE ADRENALS OF RATS UNDERGOING AN ABDOMINAL OPERATION

Group	No. of Rats	Treatment			Killed at	Adrenal Ascorbic Acid. Mean ± S.E. of Mean
		At Zero Hr.	At 30 min.	At 3 hr.		
1	7	Chlorpromazine, 10 mg./kg.	—	—	4½ hr.	321 ± 9·6
2	6	—	—	Urethane followed by operation	4½ ,,	212 ± 9·5
3	7	Chlorpromazine, 10 mg./kg.	—	Urethane followed by operation	4½ ,,	218 ± 12·4
4	6	0·9% NaCl	—	—	2 ,,	316 ± 12·4
5	6	Chlorpromazine, 15 mg./kg.	—	—	2 ,,	225 ± 10·0
6	6	Chlorpromazine, 15 mg./kg.	Urethane followed by operation	—	2 ,,	236 ± 6·9

Chlorpromazine was injected subcutaneously as 0·1 or 0·15% solution in saline.
The differences between the ascorbic acid values of Groups 2, 3, 5, and 6 are not significant.

When operation and chlorpromazine were combined (Group 3), the value was 218 mg./100 g.; thus no protection had been afforded the adrenals by preceding the operative stress with chlorpromazine.

In order to obviate the objection that the dose was too low or the timing inadequate, we examined the effect of increasing the dose to 15 mg./kg. and shortening the interval between injection and operation to 30 min. The mean ascorbic acid concentration of Group 5 (chlorpromazine alone) was 225 mg./100 g. Practically the same figure was obtained in Group 6, in which chlorpromazine was combined with the operation. Nearly the same value had been found in Group 2, subjected to the operation only. Saline controls (Group 4) gave a mean ascorbic acid content of 316 mg./100 g. Obviously, the higher dose of chlorpromazine itself acted as a stress of about the same severity as the operation. There is no simple additive effect of multiple stressing stimuli on the depression of adrenal ascorbic acid. Thus the fact that the combination of drug injection and operation, each of which are about equally potent in releasing ACTH, does not produce a greater fall in ascorbic acid than each procedure alone cannot be taken as proof—though it may suggest—that the injection has rendered the organism less susceptible to the effect of the operation.

Though inhibition by chlorpromazine of ACTH release by operative stress could not be demonstrated, the possibility remained that, perhaps as a result of the adrenolytic properties of the drug, release of ACTH in response to administration of adrenaline might be diminished. This possibility was examined in the next series of experiments. A special precaution was, however, necessary. It will be seen from Table II that the rats which had been given a subcutaneous injection of saline (Group 4) showed an adrenal ascorbic acid content of 316 mg./100 g., which is nearly 25% below normal. This response to the injection of saline, which is an effect of emotion, was undesirable in experiments in which conscious animals were to be subjected to several subcutaneous injections. It can be abolished by accustoming the rats to injections; this was done, in all rats used in the next experiment, by injecting 0.9% NaCl twice daily for a week before carrying out the final test with drugs. Table III shows the results. The duration of all experiments was 4½ hr. The "trained" rats, killed 4½ hr. after an injection of saline, had an adrenal ascorbic acid content of 409 mg./100 g.; when the saline was replaced by 10 mg./kg. chlorpromazine, there was a fall to 323 mg. When, 2½ hr. after the chlorpromazine, adrenaline (200 μg./kg.) was injected, there was a further significant fall in ascorbic acid; if the initial injection of chlorpromazine was omitted, the response to the same dose of adrenaline was a little but not significantly larger. It follows that a dose of chlorpromazine, large enough to cause some release of ACTH by itself, is nevertheless unable to inhibit appreciably the pituitary response to a dose of adrenaline administered 2½ hr. later, at a time when, to judge from the fall in rectal temperature, some, at least, of the actions of chlorpromazine are still present.

TABLE III

ASCORBIC ACID (MG./100 G. FRESH ADRENAL) IN THE ADRENALS OF "TRAINED" RATS INJECTED SUBCUTANEOUSLY WITH DRUGS AND KILLED AT 4¼ HR.

Group	No. of Rats	Treatment	Adrenal Ascorbic Acid. Mean±S.E. of the Mean
1	11	0·9% NaCl at zero hr. ..	409±19
2	23	Chlorpromazine at zero hr. ..	323±16
3	23	Chlorpromazine at zero hr. Adrenaline 200 μg./kg. at 2½ hr.	286± 7·8
4	23	Adrenaline 200 μg./kg. at 2½ hr.	270± 7·2

Chlorpromazine (10 mg./kg.) was injected as 0.1% solution in 0.9% NaCl; the same volume of 0.9% NaCl without chlorpromazine was injected into the control rats of Group 1.

The differences between Groups 1 and 2, 2 and 3, 2 and 4 are significant ($P < 0.01$), but not that between 3 and 4 ($P < 0.1$, > 0.05).

DISCUSSION

The clinical picture of morphine poisoning in the cat is modified by premedication with chlorpromazine; actions of chlorpromazine, such as muscular weakness and relaxation of the nictitating membranes, occur side by side with a somewhat damped manifestation of the effects of morphine —salivation, vomiting, tremors, excitement, and, very occasionally, convulsions. The characteristic central sympathetic stimulation by morphine is not inhibited by chlorpromazine. In contrast, premedication with a true morphine antagonist, nalorphine, abolishes all clinical signs of morphine poisoning, and prevents the central sympathetic stimulation which results in a fall in hypothalamic noradrenaline and in a depletion of amines from the stores of the innervated adrenal gland.

The observation that a peripheral sign of circulating medullary amines, mydriasis, occurred in the denervated pupil of cats treated with chlorpromazine and morphine led to the revision of the view that the peripheral adrenolytic action of chlorpromazine need last for many hours. Confirmation was obtained by observations on the dog, in which no vestige of adrenolytic action was

found 140 min. after 4.5 mg./kg. chlorpromazine given intravenously, and partial recovery had occurred much earlier.

In the French clinical literature, chlorpromazine is sometimes classed among the ganglion-blocking agents (Laborit and Huguenard, 1951). In their experimental study, Courvoisier *et al.* (1953) demonstrated that large doses have a mild inhibitory action on transmission in the vagal ganglia of the heart. No corresponding effect in the sympathetic system was found by Reuse (1954), who compared the effects on the nictitating membrane of stimulating the preganglionic and the postganglionic fibres of the superior cervical ganglion of the cat. In complete agreement with our results on the dilatation of the dog's pupil, Reuse found that even large doses of chlorpromazine did not impair transmission in the ganglion. Reuse also showed that sympatholytic in contrast to adrenolytic action required very high doses (above 5 mg./kg.) of chlorpromazine, and the same observation was made by us in the dog.

The experiments on rats, intended to investigate whether adrenocortical response to stress was inhibited by chlorpromazine, failed to demonstrate such an action; it was, however, necessary to choose a dosage and timing that would minimize interference by the release of ACTH caused by chlorpromazine itself. If no more than 10 mg./kg. were administered and 4½ hr. allowed to elapse between injection of drug and ascorbic acid estimations, some depletion of adrenal ascorbic acid was caused by the chlorpromazine, but not enough to obscure the greater depletion due to the operative stress. This dose, given 3 hr. before the operation, did not inhibit the adrenal response to the second stress. It may be argued that larger doses would have produced such an inhibition; but, as they cause a depletion of adrenal ascorbic acid equal to that of the operation, the determination of the share to be attributed to each stressing agent when both are superimposed is impossible, and interpretation of the results would necessarily be ambiguous. Recently, Georges and Cahn (1953) have also come to the conclusion that administration to rats of a "lytic cocktail" containing approximately 11 mg./kg. chlorpromazine did not prevent the release of ACTH in operative stress, provided the body temperature was above 27° C. Contrary claims by Aron, Chambon and Voison (1953), who used doses of chlorpromazine of 10–50 mg./kg. in order to prevent the adrenal ascorbic acid fall caused by unilateral adrenalectomy, are possibly due to the use of very small numbers of rats and the lack of unoperated controls treated with the drug alone. Georges and Cahn (1953) describe a fall in eosinophils 4 hr. after an injection of 22 mg./kg. chlorpromazine, and Filk and Loeser (1954) report a loss of adrenal lipids a few hours after feeding chlorpromazine. Both phenomena indicate a release of ACTH in response to this drug.

It seemed possible that, even if chlorpromazine did not prevent operative stress from releasing ACTH, it might prevent adrenaline from doing so, at least as a result of its peripheral adrenolytic action. Here again, dosage and timing had to be chosen so that there was only a moderate depletion of ascorbic acid by the chlorpromazine alone. Under these conditions, the results were not impressive. The ascorbic acid depletion by adrenaline alone was just very slightly greater than that caused by adrenaline after premedication with chlorpromazine, but the difference was not significant ($p<1, >0.05$) in spite of the use of large groups of rats. Whatever slight protection might have been exerted may, of course, be entirely due to the inhibition of the peripheral action of the adrenaline.

The foregoing experiments have not substantiated the hope, expressed by Courvoisier *et al.* (1953), that the administration of chlorpromazine might produce a kind of "chemical adrenalectomy" by inhibiting adrenomedullary secretion, or the claim by Aron *et al.* that the drug caused the equivalent of a hypophysectomy by preventing the release of ACTH. The explanation of the shock-preventing action of this drug, rather than lying in an inhibition of the adrenal defence mechanism of the organism, must be sought elsewhere —perhaps in the metabolic effects it produces.

Summary

1. Chlorpromazine (25 mg./kg. subcutaneously) does not inhibit the stimulation of the sympathetic centres produced by morphine in the cat, as judged by the following criteria: a fall in hypothalamic noradrenaline and a depletion of the stores of medullary amines in the innervated adrenal. During certain periods of the experiment, the amount of medullary amines released into the circulation dilates the (innervated and denervated) pupil and is thus sufficient to overcome the adrenolytic action of chlorpromazine. In contrast, nalorphine inhibits all manifestations of morphine poisoning including the stimulation of the sympathetic centres.

2. In the dog, the duration of the antiadrenaline effect of chlorpromazine was followed, and its

alleged inhibitory action on ganglionic transmission was examined ; no such action was found on the superior cervical ganglion.

3. The release of ACTH in operative shock was not prevented in the rat by chlorpromazine (10 mg./kg.) administered 3 hr. before the operation ; nor was the release of ACTH by adrenaline (200 μg./kg. subcutaneously) prevented. Chlorpromazine itself caused some release of ACTH at this dose level ; if the dose was increased to 15 mg./kg., the release of ACTH was of the same magnitude as that caused by the operation, or by an injection of adrenaline ; a satisfactory investigation of a possible inhibitory effect of such doses of chlorpromazine on the ACTH releasing power of other procedures was thus not possible.

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THE RELEASE OF CORTICOTROPHIN DURING SEVERE STRESS IN THE RAT TREATED WITH PENTOBARBITONE AND MORPHINE*

By

Margarethe Holzbauer and Marthe Vogt

Pretreatment with morphine was shown by *Briggs & Munson* (1955) to prevent the fall in adrenal ascorbic acid elicited in young rats by dissection of the bile duct. The operation, which took 2 min. to perform, caused maximal loss of adrenal ascorbic acid in rats deeply anaesthetized with pentobarbitone, but morphine (20 mg./kg.), injected 10 min. after the barbiturate, completely prevented this fall. Proof that it was the secretion of corticotrophin (ACTH) which was inhibited and not the response of the adrenal cortex to released ACTH, was obtained by demonstrating normal ascorbic acid depletion in hypophysectomized rats pretreated with pentobarbitone and morphine and then given ACTH.

Since no direct means are at present available of estimating the 'resting' adrenocortical secretion of an unstressed rat, it appeared worth while to test whether the combined administration of pentobarbitone and morphine might provide such a method. If, by protecting the rat with morphine, the operation of cannulating a renal vein for the collection of adrenal vein blood could be performed without causing any release of ACTH, resting values for corticosterone secretion might be obtained by analysing the adrenal effluent. The following experiments examine this possibility.

METHODS

Male white rats weighing between 250 and 300 gm. were used. The operation was intended closely to mimic the manipulation and blood loss involved in the collection

* Herrn Prof. Dr. R. Rigler zum 60. Geburtstag gewidmet.

of adrenal vein blood. It consisted of the introduction of cannulae into trachea and femoral vein, a midline abdominal incision, the intravenous injection of 2 mg. heparin and the ligation of the left adrenal vein which was then cut distally in order to allow free flow of adrenal blood into the abdominal cavity. The operative procedures took 5–10 min., and bleeding from the adrenal vein was allowed to proceed for 15 min. The rat was then decapitated and the adrenals were dissected, weighed, and ground in trichloracetic acid for the estimation of ascorbic acid by the method of *Roe & Kuether* (1943).

The injections preceding the operation were all made intraperitoneally and sterile precautions taken when repeated injections were given. In order to avoid any fall in ascorbic acid by environmental disturbances, the animals were housed in individual cages and kept in a thermostatically controlled room in which also the final experiment was performed. Weighing, injecting and feeding were done by the same experimenter. Timing of the injections of pentobarbitone sodium and morphine HCl on the day of the experiment followed the directions of *Briggs & Munson* (1955), pentobarbitone being injected at zero time, morphine HCl (20 mg./kg.) at 10 min., and the operation started at 20 min. The doses of pentobarbitone will be discussed in the text. The volumes injected were 2 ml./kg. throughout. The solutions were brought to body temperature before injection.

RESULTS

Since any handling, including injections of physiological salt solutions, causes some fall in adrenal ascorbic acid, all the rats used in this work were 'trained' by a preliminary series of daily injections. Rats which, in the final experiments, were given morphine, were trained and desensitized by injections of morphine (20 mg./kg. daily), and all remaining rats by injections of 0.9 % NaCl. The period of training lasted between 6 and 13 days.

Fig. 1 shows that the treatment with morphine and pentobarbitone (45 mg./kg.) by itself (Group 1), in spite of the preliminary desensitization, caused an equally large loss of ascorbic acid as the combination of pentobarbitone and operation (Group 2); when morphine was given as well, the fall was bigger still (Group 3). This was very puzzling and could either be due to a persistance of the action of morphine or to an unexpected action of the pentobarbitone. Thus, in the groups represented in Fig. 2, no operations were performed, but the effects of morphine and pentobarbitone examined by themselves. The results exonerate the morphine, which is shown to be completely inactive in rats pretreated with morphine (compare Groups 8 and 7), but incriminate the pentobarbitone, which, when given in a dose of 50 mg./kg., caused a depletion of ascorbic acid (Group 5). This dose had been chosen since, in combination with morphine, a larger dose of pentobarbitone might be lethal; for this very reason *Briggs & Munson* (1955) used even less, 40 mg./kg. At this dose level, however, our rats still reacted to touch, and an increase to 55 mg./kg. was necessary in order to produce complete anaesthesia. With the higher dose, there was no ascorbic acid depletion by the anaesthetic alone (Group 6). In the third

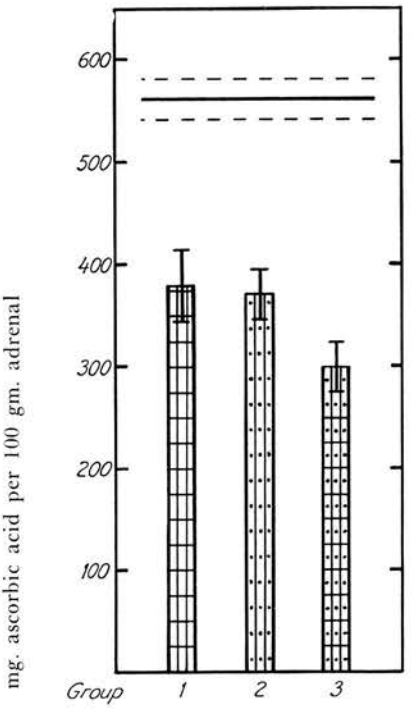

Fig. 1.
Adrenal ascorbic acid concentration (mean ± S. E. of the mean) of 3 groups of 6 rats decapitated 45 min. after »zero time«.

Group	Pretreatment	At 0 time	10 min.	20 min.
1	daily injections of 1 % M. for 13 days	P., 45 mg./kg.	M.	–
2	daily injections of 0.9 % NaCl for 13 days	P., 55 mg./kg.	–	Op.
3	daily injections of 1 % M. for 13 days	P., 45 mg./kg.	M.	Op.

P., injection of pentobarbitone Na. M., injection of morphine HCl (20 mg./kg.). Op. = operation.

The continuous horizontal line represents the mean ascorbic acid concentration in the adrenals of a group of control rats which were not handled at all on the day of the experiment. The broken line is the S. E. of this mean.

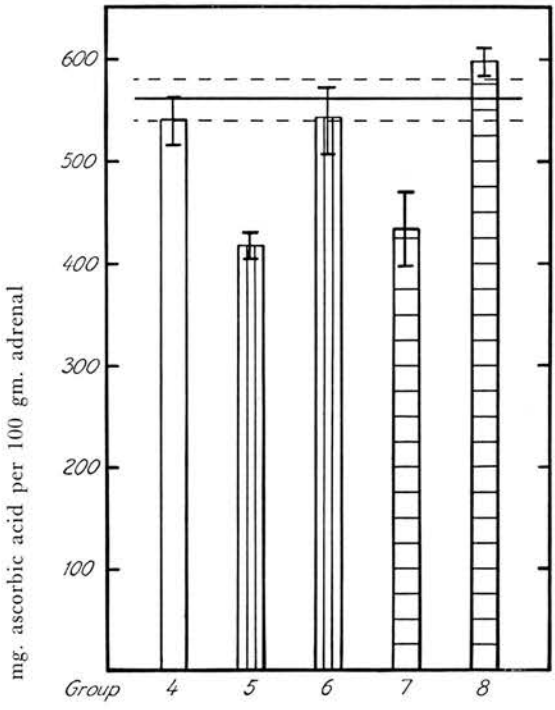

Fig. 2.
Adrenal ascorbic acid concentration (mean ± S. E. of the mean) of 5 groups of 8 rats decapitated 45 min. after »zero time«.

Group	Pretreatment	At 0 time	10 min.
4	daily injections of 0.9 % NaCl for 8 days	NaCl	–
5	daily injections of 0.9 % NaCl for 8 days	P., 50 mg./kg.	–
6	daily injections of 0.9 % NaCl for 6 days	P., 55 mg./kg.	–
7	daily injections of 0.9 % NaCl for 6 days	–	M.
8	daily injections of 1 % M. for 6 days	–	M.

For meaning of P., M. and horizontal lines see Fig. 1.

experiment (Fig. 3), all rats were first desensitized to morphine, and all were given the larger dose of pentobarbitone; only one of the rats died of respiratory failure before the end of the experiment. The adrenal ascorbic acid was only

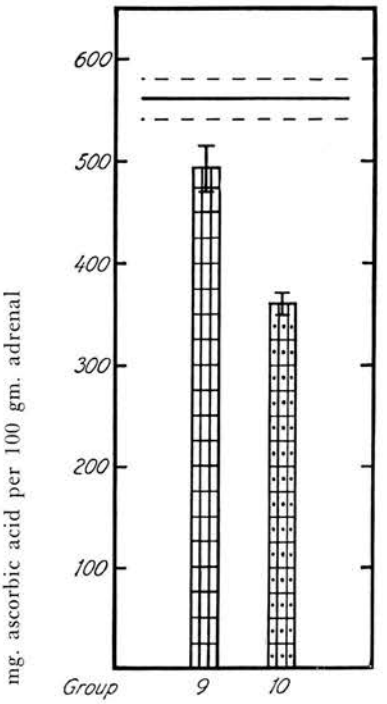

Fig. 3.
Adrenal ascorbic acid concentration (mean ± S. E. of the mean) of 2 groups of 8 rats decapitated 45 min. after »zero time«.

Group	Pretreatment	At 0 hr.	10 min.	20 min.
9	daily injections of 1 % M. for 6 days	P., 55 mg./kg.	M.	–
10	daily injections of 1 % M. for 7 days	P., 55 mg./kg.	M.	Op.

For meaning of P., M. and horizontal lines see Fig. 1.

just significantly lowered by the drugs (Group 9), but was greatly reduced when drugs and operation were combined. Thus protection from the stress of the operation was not achieved.

On comparing the adrenal ascorbic acid content of glands in our animals with that found in earlier work or by other workers (e. g. *Holzbauer & Vogt*, 1954; *Briggs & Munson*, 1955), it is obvious that the depletion was less severe. This, however, is not an effect of the drugs but of the timing. The ascorbic

acid was estimated 25 min. after the beginning of the operation, whereas it is usual to allow 1–4 hr. between start of stress and removal of the adrenals in order to obtain a maximal fall in ascorbic acid. In the present work the time was made as brief as possible; by thus shortening the duration of the stress we hoped to obtain the most favourable conditions for a suppression of the release of ACTH.

DISCUSSION

The experiments fully confirm Briggs' and Munson's observation that adrenal ascorbic acid depletion by morphine is easily prevented by pretreating the rats with one daily injection of morphine for 6 or more days. They further show that pentobarbitone by itself, given to rats desensitized to the procedure of intraperitoneal injections, causes a fall in ascorbic acid if full anaesthesia is not obtained. *Briggs & Munson* (1955) comment on the necessity of selecting deeply anaesthetized animals in order to prevent the release of ACTH by operation, but it was not apparent from their work that an injection of pentobarbitone as such acted as a stress if deep anaesthesia was not attained. Recent work of *Sayers* (1957) has shown that ether anaesthesia produces initially a large release of ACTH, followed by a period of normal or subnormal secretion. The assumption that, in contrast, pentobarbitone achieves anaesthesia without an initial phase of stimulation of the pituitary gland is seen to be only partially correct and to depend on the use of a dose which will rapidly produce deep surgical anaesthesia. We would probably not have encountered this phenomenon, if we had not worked with a strain of rats which was rather insensitive to pentobarbitone and required larger doses than those recommended by Briggs and Munson for the production of full anaesthesia.

Finally, it follows from these experiments that the response of the pituitary gland to an operation of the severity required for collecting adrenal vein blood, is not prevented by the combination of pentobarbitone and morphine which is sufficient to suppress the response to dissection of the bile duct. There are several possibilities which might explain the difference in the results. The first and simplest is the fact that the collection of adrenal vein blood is a much more damaging procedure, involving, as it does, not only operative procedures lasting 10 instead of 2 min., but also a 15 min. period of haemorrhage.

Briggs & Munson (1955) have shown that the influence of morphine on the release of ACTH by histamine depends on the dose of histamine, the response to 10 mg./kg. being suppressed, that to 30 mg./kg. diminished and that to 300 mg./kg. remaining unaffected. Since morphine was used in the largest tolerated dose only the damaging agent could be graded. Similarly, milder operative procedures may yield to the influence of morphine whereas severer ones may not. The great severity of the stress during collection of adrenal vein

blood is evident from the observation that in these circumstances an infusion of ACTH is unable to increase cortical secretion any further (*Holzbauer & Vogt*, 1957). It is interesting that quite profound depression of the central nervous system by the combination of general anaesthesia with morphine does not appear to affect even the speed of activation of the anterior lobe of the pituitary gland if the challenge is sufficiently great.

SUMMARY

Suppression of the release of corticotrophin by pentobarbitone and morphine (*Briggs & Munson*, 1955) was attempted in rats in which adrenal vein blood was being collected for 15 min.

Corticotrophin release, as indicated by a fall in the adrenal ascorbic acid, was neither inhibited, nor appreciably delayed, under circumstances which are known, in the absence of morphine, to produce adrenocortical secretion of maximal intensity.

Whereas it was confirmed that doses of pentobarbitone sodium which rapidly lead to full anaesthesia do not cause a release of corticotrophin, slightly smaller doses released corticotrophin in the absence of any further stimulus. This was not due to the handling required for the injections, since all rats used in this work had been 'trained' by a preliminary series of daily intraperitoneal injection of 0.9 % NaCl.

ACKNOWLEDGMENTS

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Metabolism of 17α-Methylandrostenediol and 17α-Methyltestosterone by the Rat Adrenal Gland *in Vitro*

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ABSTRACT. Rat adrenal tissue *in vitro* oxidized 17α-methylandrost-5-ene-3β, 17β-diol (MAD) to 17α-methylandrost-4-en-17β-ol-3-one (17α-methyltestosterone). Both MAD and 17α-methyltestosterone were further metabolized to at least 5 ultraviolet light-absorbing products. Evidence was obtained for the identification of the major product as 11β-hydroxy-17α-methyltestosterone. The next most abundant product was probably 11-oxo-17α-methyltestosterone. The other products were formed in lesser amounts and little progress was made in their identification. The metabolism of 17α-methyltestosterone to these metabolites was strongly inhibited by the addition of Metopirone to the incubation medium, suggesting that hydroxylation is a major pathway of metabolism. (*Endocrinology* **81:** 1278, 1967)

IN 1959, Saffran and Vogt (1) observed that the administration of MAD[6] to rats decreased the secretion of corticosterone in adrenal venous blood. The experiments described in this paper present evidence for the oxidation of MAD to 17α-methyltestosterone and for the further metabolism of 17α-methyltestosterone by the rat adrenal gland *in vitro*. The effects of MAD and 17α-methyltestosterone on the formation of steroids by the rat adrenal *in vitro* will be described elsewhere.

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[6] Abbreviations and trivial names used in this paper: MAD = 17α-methylandrostenediol = 17α-methylandrost-5-ene-3β, 17β-diol; 17α-methyltestosterone = 17α-methylandrost-4-en-17β-ol-3-one.

Materials and Methods

Adrenals. In most experiments, young adult male rats derived from the Sprague-Dawley strain (Canadian Breeding Laboratories, St. Constant, Que.), weighing in the range of 150 to 250 g, were used. Rats of relatively uniform body weight (±10 g) were used in each experiment. The rats were anesthetized with an intraperitoneal injection of 4 mg/100 g body weight of sodium pentobarbital and were decapitated. The adrenal glands were removed, freed of adhering fat and carefully quartered with fine scissors.

Incubation. The 8 adrenal quarters from each rat were distributed among 8 flasks containing Krebs-Ringer bicarbonate medium with 0.2% glucose. The amount of tissue and volume of medium varied according to circumstances of the experiment but usually 15 mg of tissue in 1.5–3.0 ml of medium was placed in each flask. The tissue was preincubated in an atmosphere of 95% O_2-5% CO_2 in a Dubnoff shaking incubator at 37 C for 30–60 min. The preincubation medium was discarded and replaced with fresh medium for the incubation period of about 2 hr. Steroids were added to the incubation

TABLE 1. Formation of ultraviolet absorbing lipids by rat adrenal tissue incubated with MAD and ACTH

Additions	μmole (corticosterone equivalents)/100 mg adrenal tissue/2 hr	Difference due to MAD
None	0.09 ±0.02	
MAD	0.21 ±0.02	0.12
ACTH	0.29 ±0.02	
ACTH+MAD	0.42 ±0.08	0.13

Mean of 4 experiments ±SE.
Volume of medium, 1.5 ml; tissue weight, approx 15 mg/flask; gas phase, 95% O_2/5% CO_2; temperature, 37 C; preincubation period, 50 min; incubation period, 120 min; MAD, 0.5 μmole in 10 μl 95% ethanol; control, 10 μl 95% ethanol; ACTH 100 mU in 50 μl water.

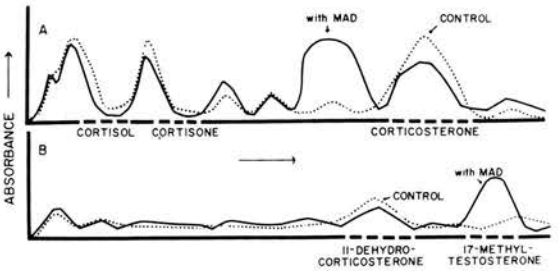

FIG. 1. A. Ultraviolet scan of paper chromatogram, developed in the toluene/propylene glycol system, of a methylene chloride extract of the medium in which rat adrenal tissue was incubated with and without MAD under conditions similar to those of Table 1. The mobility of standard steroids is shown by a dashed portion of the abscissa below the scan. B. Ultraviolet scan of the runoff of the chromatogram in A.

medium as small volumes (about 10 μl) of ethanolic solutions. The same volume of ethanol was added to control tissues. ACTH, when used, was added as a small volume of aqueous solution.

Extraction. The incubation was terminated by decanting the medium from the tissue into a glass-stoppered test tube. The incubation flask was rinsed with an equal volume of water and the rinse was added to the test tube. The pooled medium and rinse was then extracted twice with equal volumes of methylene chloride; the tubes were centrifuged after each extraction to separate the phases. The methylene chloride layer was transferred to a fresh tube with a syringe and long needle; the syringe and needle were rinsed into the fresh tube with methylene chloride. The methylene chloride was then evaporated in a stream of nitrogen, or, in some cases, *in vacuo* in a rotary evaporator.

Paper chromatography. The following paper chromatographic systems were used: toluene/propylene glycol on Whatman No. 42 paper (2); benzene/propylene glycol on Whatman No. 1 paper (3); cyclohexane:benzene (1:1)/propylene glycol on Whatman No. 1 paper (3); benzene/methanol:water (1:1) on Whatman No. 4 paper (4); isooctane:benzene (1:2)/methanol:water (1:1) on Whatman No. 1 paper (5). Materials were eluted from the paper strips in the apparatus described by Saffran and Sharman (6) with ethylacetate:methanol (2:1) or by soaking the shredded paper in methanol overnight. Ultraviolet light absorbing areas were detected either by direct observation under UV light (λ max = 256) or by scanning the paper strip in a Photovolt Densicord equipped with a UV light source. The ultraviolet light absorbing areas were eluted as described above and the absorbance was measured in a Beckman model DU spectrophotometer.

Absorption spectra. Ultraviolet absorption spectra were taken on the Beckman model DK-2 recording spectrophotometer to detect and measure substances containing the Δ^4-3-oxo group. The same instrument was used to record sulfuric acid spectra according to the procedure of Bernstein and Lenhard (7).

INH reaction. The reaction with isonicotinic acid hydrazide (INH) was also used to detect Δ^4-3-oxo-containing compounds (8).

Formation of derivatives. The method of Mattox, Mason and Albert (9) was used for acetylation of hydroxyl groups under mild conditions. For more prolonged acetylation, the method of Berliner and Salhanick (10) was used. The method of Balant and Ehrenstein (11) was followed for saponification. The method described by Nowaczynski *et al.* (12) was used for the oxidation of hydroxyl groups with chromic acid.

Detection and measurement of radioactive isotopes. Paper chromatograms were scanned in a Vanguard gas-flow scanner, model 880, or in a Packard Radiochromatogram Scanner, model 7200, to locate radioactive zones. Quantitative determinations of radioactivity of eluates of these zones were made on a Packard model 314E Tri-Carb scintillation spectrometer.

Steroids. MAD was obtained as gifts from Organon Ltd., Newhouse, Scotland, and Charles

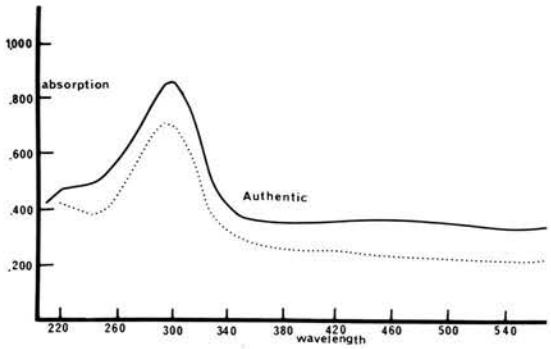

FIG. 2. Sulfuric acid spectra of authentic 17α-methyltestosterone (——) and the material in the eluate of the zone with the mobility of 17α-methyltestosterone (. . .) in the chromatogram illustrated in Fig. 1B.

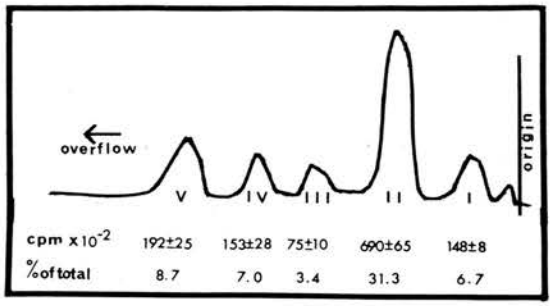

FIG. 3. Radioactive scan of paper chromatogram developed in cyclohexane: benzene/propylene glycol of a methylene chloride extract of the medium in which rat adrenal tissue was incubated with approximately 5.6 μg (2.2×10^5 cpm) of 4-^{14}C-17α-methyltestosterone, added in 10 μl of 95% ethanol. The bottom row of figures represents the percentage of the total added radioactivity recovered under each of the peaks.

E. Frosst and Company, Montreal. 17α-Methyltestosterone was also obtained from Charles E. Frosst and Company. 11α-Hydroxy, 11β-hydroxy, 11-oxo, 7α-hydroxy, 9α-hydroxy, 16α-hydroxy and 6β-hydroxy derivatives of 17α-methyltestosterone were obtained from The Upjohn Company, Kalamazoo, through the kind cooperation of Dr. J. C. Babcock. SU-4885 (Metopirone) was supplied by Ciba Limited, Montreal.

4-^{14}C-labeled 17α-methyltestosterone was purchased from New England Nuclear Corporation, Boston.

All steroids were examined for purity in at least 2 chromatographic systems.

ACTH. A sample of the third International Working Standard corticotrophin was supplied by the National Institute for Medical Research, Mill Hill, London.

Results

Incubation with MAD. Rat adrenal glands incubated *in vitro* with MAD produced more ultraviolet absorbing lipid than controls, even in the presence of ACTH (Table 1). Chromatography of methylene chloride extracts of the incubation media in the toluene/propylene glycol system revealed qualitative and quantitative differences in the ultraviolet scan of the chromatogram when MAD had been added to the incubations (Fig. 1). Two new ultraviolet absorbing peaks were observed: the first had a mobility of 0.8 that of corticosterone; the second appeared in rechromatography in the same system of the overflow of the first chromatogram. The material in the second peak had the same mobility as 17α-methyltestosterone, did not separate from 17α-methyltestosterone on mixed chromatography (2), and had an ultraviolet absorption peak at 240 mμ and a sulfuric acid spectrum similar to that of authentic 17α-methyltestosterone (Fig. 2).

Ultraviolet scans virtually identical with those in Fig. 1 were obtained with chromatograms in experiments in which 17α-methyltestosterone was substituted for MAD.

Metabolism of 4-^{14}C-17α-methyltestosterone. The medium of rat adrenals incubated in the presence of 5.6 μg ($= 2.2\times10^5$ cpm) of 4-^{14}C-17α-methyltestosterone was extracted with methylene chloride and the extract was chromatographed in the cyclohexane: benzene/propylene glycol system. Two radioactive zones were located by scanning. The less polar zone had the mobility of 17α-methyltestosterone and probably represented unchanged substrate. The material in the more polar zone was eluted and rechromatographed in benzene/propylene

TABLE 2. Recrystallization of metabolites II and V of 17α-methyltestosterone to constant radioactivity after addition of carrier 11β-hydroxy-17α-methyltestosterone and 11-oxo-17α-methyltestosterone, respectively

Sample	Crystals			Mother liquor specific activity cpm/mg
	Specific activity, cpm/mg		mp	
	Calculated	Observed		
Compound II-^{14}C (13,540 cpm) +13 mg 11β-OH-17α-methyltestosterone	1,040		213–215	
1st crystallization		990	214–215	
2nd crystallization		1,040	215	
3rd crystallization		990	215	
Compound II-^{14}C (80,500 cpm) +5 mg 11β-OH-17α-methyltestosterone	16,100			
1st crystallization		16,400		17,800
2nd crystallization		16,100		13,900
3rd crystallization		16,000		17,000
4th crystallization		16,250		16,050
Compound II-^{14}C after chromic acid oxidation (8700 cpm) +10 mg 11-oxo-17α-methyltestosterone	870		174	
1st crystallization		820	173	
2nd crystallization		860	173	
3rd crystallization		840	174	
Compound V-^{14}C (10,320 cpm) +10 mg 11-oxo-17α-methyltestosterone	1,030		173	
1st crystallization		980	173	
2nd crystallization		950	174	
3rd crystallization		1,010	173	

glycol. Five radioactive zones were detected in addition to a small amount at the origin (Fig. 3). No radioactivity was detected in the overflow.

Major metabolites. The major metabolite (peak II) was rechromatographed in isooctane:benzene/methanol:water and then in benzene/propylene glycol for 36 hours. A single radioactive peak was detected each time. The mobility in both systems was the same as that of authentic 11β-hydroxy-17α-methyltestosterone.

Oxidation of the material in peak II by chromic acid yielded material with the mobility of 11-oxo-17α-methyltestosterone in the above two chromatographic systems. The material in peak V exhibited the same mobilities in benzene/propylene glycol and in cyclohexane:benzene/propylene glycol as either 11-oxo-17α-methyltestosterone or the oxidation product of peak II. The materials in peaks II and V were not acetylatable by the methods used. The mobility of the material in peak V was not altered by treatment with chromic acid.

The radioactivity of the purified material in peak II was determined; then the material was mixed with carrier 11β-hydroxy-17α-methyltestosterone, and was recrystallized at least three times by adding a concentrated solution in acetone to an excess of isooctane and allowing the mixture to stand overnight in the refrigerator. The specific activity of the crystals remained constant. Similarly, the oxidation product of material II was mixed with carrier 11-oxo-17α-methyltestosterone, recrystallized, and the specific activity of the crystals was estimated; the material in peak V was also mixed with the same carrier and was recrystallized. The melting points of

the crystals were determined at every stage. These results (Table 2) show a constant specific radioactivity of the materials through at least three recrystallizations, as well as constant melting points.

A large amount of material II was accumulated by a four hour incubation of 96 rat adrenal glands in 12 vessels with a total of 2.4 mg of 17α-methyltestosterone and a tracer amount of ^{14}C-methyltestosterone. At the end of the incubation, the lipids in the media of the 12 vessels were extracted with methylene chloride, and chromatographed in the Bush system. The zone with the mobility of 11β-hydroxy-17α-methyltestosterone also contained some of the corticosterone formed during the incubation. Therefore, the material eluted from this zone was acetylated and the reaction mixture was chromatographed in cyclohexane:benzene/propylene glycol to separate corticosterone acetate from the material in the 11β-hydroxy-17α-methyltestosterone zone. The material in this zone was eluted from the paper and recrystallized twice from acetone:isooctane. The yield was about 400 μg. The crystals melted at 210–213 C. There was no depression of the melting point on mixture with authentic 11β-hydroxy-17α-methyltestosterone (mp 213 C).

Minor metabolites. The materials in peaks I, III and IV were formed in lesser amounts and only limited progress was made in characterizing them. The materials in all three peaks absorbed ultraviolet light, with maxima between 238 and 240 mμ, gave a positive INH reaction, and had absorption spectra in concentrated sulfuric acid with peaks at 280–290 mμ, all evidence for the presence of the Δ^4-3-oxo group.

Material I. Material I was relatively polar. It was acetylated only by the method of Berliner and Salhanick (10) to a less polar product. The mobility of material I differed from those of 11α-hydroxy-17α-methyltestosterone, 16α-hydroxy-17α-methyltestosterone and 9α-hydroxy-17α-methyltestosterone. However, material I had the same mobility as 6β-hydroxy-17α-methyltestosterone in the benzene/propylene glycol system. Therefore, material I and 6β-hydroxy-17α-methyltestosterone were separately treated with KOH in ethanol in an attempt to rearrange the Δ^4-3-oxo-6β-hydroxy compound into the tautomeric 3,6-dioxo form (13, 14). The authentic 6β compound lost absorption at 240 mμ, and acquired a peak at 260 mμ; after heating, a second peak appeared at 380 mμ. On the other hand, the absorption spectrum of material I was unchanged by ethanolic KOH. The 3,6-dioxo form of the authentic 6β compound was readily separated from the KOH-treated material I by chromatography in benzene/propylene glycol. The 3,6-dioxo compound was detected by dipping the chromatogram in a solution of 2,4-dinitrophenylhydrazine, and material I was located by its radioactivity.

Material III. Material III could be oxidized and acetylated (10); unchanged acetate was recovered after treatment of the acetate with chromic acid.

Material IV. Material IV resisted acetylation and oxidation by the procedures used. Materials III and IV had chromatographic mobilities that differed from the reference standards mentioned above.

Effect of SU-4885. Metopirone (SU-4885) was added to rat adrenal glands *in vitro* along with trace amounts of 4-^{14}C-17α-methyltestosterone. The conversion of 17α-methyltestosterone to all five metabolites was reduced; the formation of material II was inhibited most (Table 3).

Discussion

The work described in this paper suggests that the rat adrenal cortex oxidized MAD to 17α-methyltestosterone and that 17α-methyltestosterone was hydroxylated to the 11β-hydroxy compound, which was,

TABLE 3. Effect of SU-4885 on the metabolism of 4-^{14}C-17α-methyltestosterone by rat adrenal glands *in vitro*

	Adrenal tissue mg/flask	Unconverted 17α-methyl-testosterone	I	II	III	IV	V	Total radioactivity recovered from all zones
		cpm $\times 10^{-3}$ per zone						
Controls	26.9 ±1.2	94 ±4.9	15 ± .8	68 ±6.5	8 ±1.0	15 ±2.8	19 ±2.5	219
SU-4885	28.0 ±0.9	206 ±6.3	4 ±0.5	1 ±0.3	2 ±0.5	3 ±2.2	10 ±1.1	226

Average of 4 experiments ±SE.
4-^{14}C-17α-methyltestosterone, 7μg = 250 $\times 10^3$ cpm/flask.
SU-4885, 50 μg/flask.
See Table 1 for other conditions of the experiment.

in turn, partially oxidized to the 11-oxo compound. Other metabolites were formed, but these have not yet been identified. Two of these (I and III) probably contained hydroxyl groups at position(s) other than 6β, 7α, 9α, 11α and 16α. Another (IV) had no readily acetylatable groups. In addition to the five major metabolites, other minor ^{14}C-labeled material appeared in the very polar region near the origin of the chromatograms.

It is conceivable that 17α-methyltestosterone was metabolized by adrenal enzymes normally acting in the biosynthesis of corticoids. Therefore, 11β-hydroxylation is not unexpected. The rat adrenal also actively hydroxylates the C-18 methyl group to form 18-hydroxy-11-deoxycorticosterone (16, 17) and 18-hydroxycorticosterone (18). Therefore, one might guess that one or more of the unidentified adrenal metabolites of 17α-methyltestosterone would possess a hydroxyl group at C-18.

More indirect evidence for the identity of the metabolites of 17α-methyltestosterone was obtained from the experiment in which SU-4885 suppressed the formation of 11β-hydroxy-17α-methyltestosterone, because SU-4885 is well known to inhibit 11β-hydroxylation (18). However, SU-4885 has also been shown to inhibit hydroxylation at C-18 (19); in the present work, SU-4885 suppressed the formation of other metabolites of 17α-methyltestosterone as well.

The metabolism of testosterone by the adrenal cortex has been studied by others. Hydroxylation at C-11 has been reported after incubation with beef adrenal homogenates (20), human adrenal homogenates (21) and human adrenal cortex slices (22). Reduction of Ring A of testosterone has not been reported, but oxidation of the C-17β-hydroxyl group to yield androst-4-ene-3,17-dione and the corresponding 11β-hydroxylated and 11-oxo compounds has been shown by Chang *et al.* (21). Except for oxidation of the 17β-hydroxyl group, the metabolism of 17α-methyltestosterone by the adrenal cortex has been shown to be similar to the metabolism of testosterone by the same gland.

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